14-3-3 ϵ functions as an oncogene in SGC7901 gastric cancer cells through involvement of cyclin E and p27^{kip1}

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Abstract. Investigation into the highly conserved 14-3-3 ϵ protein has become increasingly important in cell biology due to its involvement in cell survival signaling, cell cycle control and apoptosis. The 14-3-3 ϵ protein has been found to exert an impact on the development of various tumor types. However, the functional role and the possible mechanism of 14-3-3 ϵ in gastric cancer remains to be elucidated. A previous study by our group indicated a negative correlation between 14-3-3 ϵ expression levels and gastric cancer tissue differentiation and a positive correlation between 14-3-3 ϵ expression levels and tumor infiltration, lymph node metastasis and tumor, nodes and metastasis staging. In the present study, 14-3-3 ϵ suppression in the SGC7901 gastric cancer cell line was demonstrated to inhibit cell proliferation *in vitro* and tumor growth *in vivo* and the cell cycle-associated proteins cyclin E and p27^{kip1} may have contributed to this antitumor effect. The present study showed for the first time that reducing the expression of 14-3-3 ϵ may inhibit the proliferation and progression of gastric cancer and inhibition of this protein may provide a potential strategy for gastric cancer therapy in the future.

Introduction

Gastric cancer is one of the most frequent malignancies worldwide, particularly in Eastern Asia, according to estimations published in 2011 (1). The common treatment of gastric cancer is radical resection of the neoplastic tumor tissue with lymphadenectomy (2). Adjuvant radiotherapy and chemotherapy to eliminate dissemination or minimal residual tumor load have further improved prognosis. However, the five-year survival rate for advanced gastric cancer has remained poor (1,3). Thus, it is of great importance to further investigate the onset and

progression of gastric cancer and novel therapeutic targets. It has been widely accepted that the pathogenesis of gastric cancer is a complicated process (4-6) involving dysregulation of multiple genes (7-9), including oncogenes, tumor suppressors and DNA repair genes. However, the underlying molecular mechanism in gastric cancer initiation and progression requires further investigation.

The 14-3-3 proteins are a family of 28-33 kDa acidic polypeptides widely expressed in all eukaryotic organisms (10). There are seven mammalian isotypes denoted by β , γ , ϵ , σ , ζ , τ and η , and every subtype is encoded by a distinct gene. Due to specific phospho-serine and phospho-threonine binding activity, 14-3-3 proteins are able to interact with numerous different proteins and regulate diverse cellular biological activities, including cytoskeleton configuration, signal transduction, metabolism, differentiation, transcription and tumorigenesis (11). The 14-3-3 ϵ isoform is the most conserved member of the 14-3-3 family, with conserved sequences from plants, yeast and mammals (12). This isoform has been proposed as a candidate tumor promoter in lung cancer, breast cancer, hepatic cellular cancer, vulvar squamous cell carcinoma, follicular and papillary thyroid tumors and meningioma (12-17). However, the function and possible mechanism of 14-3-3 ϵ in gastric cancer remain unknown.

RNA interference (RNAi) is a general mechanism of eukaryotic gene regulation and a potent strategy for specific gene silencing. The vector-based approach of short hairpin (sh) RNA interference, either as selectable plasmids or as retroviruses, has previously been demonstrated to achieve specific and persistently effective suppression of gene expression in mammalian cells (18). shRNA interference is able to continuously transcribe and generate small interfering RNA and degrade complementary messenger RNA persistently inside the cells, which have been screened by resistance selection, and is functionally similar to the process of post-transcriptional gene silencing (19-21). Preliminary experiments performed by our group indicated a negative correlation between 14-3-3 ϵ expression levels and gastric cancer tissue differentiation and a positive correlation between 14-3-3 ϵ expression levels and tumor infiltration, lymph node metastasis and tumor, nodes and metastasis staging. Furthermore, by introducing 14-3-3 ϵ and Raf-1 kinase inhibitor protein (RKIP) genes into SGC7901 cells, 14-3-3 ϵ and RKIP were demonstrated to exert an opposite effect on SGC7901 human gastric cancer cells

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by regulating the mitogen-activated protein kinase signaling pathway (22). In the present study, the functional role of 14-3-3 ϵ in human gastric cancer was examined using RNAi.

Materials and methods

Chemicals and reagents. shRNA directed against 14-3-3 ϵ , control shRNA encoding a scrambled shRNA sequence that does not result in specific degradation of any cellular message RNA, monoclonal mouse anti-14-3-3 ϵ , polyclonal rabbit anti-cyclin E and anti-p27 antibody, shRNA Plasmid Transfection Reagent and puromycin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Goat anti-mouse secondary antibody and goat anti-rabbit secondary antibody were purchased from Sigma (St. Louis, MO, USA). The enhanced chemiluminescence (ECL) system for western blot analysis was provided by Amersham Pharmacia Biotech (Piscataway, NJ, USA). MTT and Giesma stain were purchased from Amresco Chemical Co., Ltd. (Solon, OH, USA). All other reagents were of molecular biology grade and purchased from Sigma (St. Louis, MO, USA) or Amersco Chemical Co. Ltd.

Cell lines and animals. The SGC7901 gastric cancer cell line was obtained from the Key Laboratory of Cancer Proteomics of the Chinese Ministry of Health, Xiangya Hospital, Central South University, China. The cells were incubated in complete RPMI-1640 (Hyclone Laboratories, Inc., Logan, UT, USA) and supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Carlsbad, CA, USA), 100 U/ml penicillin and 100 U/ml streptomycin in a humidified atmosphere at 37°C and 5% CO₂.

Female nude athymic mice, aged 4-5 weeks and each weighing ~20 g, were purchased from the Department of Experimental Zoology, Central South University (Changsha, China). The experiments were performed with the approval of the Animal Ethics Committee of Xiangya Hospital, Central South University (Changsha, Hunan, China).

Stable transfection. The cells were plated at 3x10⁵ cells per well in six-well plates. Transfections were conducted using the shRNA Plasmid Transfection Reagent according to the manufacturer's instructions. To establish stable cell lines expressing lower levels of 14-3-3 ϵ , a 1:10 passage of the transfected SGC7901 cells was performed after 48 h, followed by puromycin selection (1.5 μ g/ml). Following ~4 weeks of incubation, the resistant colonies were selected and transferred to 24-well plates. These colonies were maintained in selective culture medium with 0.75 μ g/ml puromycin. Another set of SGC7901 cells were transfected with control shRNA as an experimental control.

Western blot analysis. The cultured cells were lysed in a lysis buffer [20 mmol/l Tris (pH 7.5), 0.1% Triton X, 0.5% deoxycholate, 1 mmol/l phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin and 10 μ g/ml leupeptin] for 60 min on ice. The samples were boiled in 2X SDS sample buffer. A total of 40 μ g lysate per lane was separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Amersham Pharmacia Biotech). The membranes were blocked with 5%

non-fat dry milk in Tris-buffered saline and Tween-20 buffer for 1 h at room temperature and then incubated with the appropriate primary antibodies for 2 h at room temperature, followed by incubation with the respective peroxidase-conjugated secondary antibodies for 45 min at room temperature. Immunoreactive proteins were visualized using the ECL detection system. β -actin was detected simultaneously as a loading control. The protein bands were quantified with Image J 1.48 (National Institutes of Health, Bethesda, MA, USA) analysis. The expression of each protein was calculated using the ratio of the intensity of each protein band to that of β -actin. All experiments were performed in triplicate.

Cell proliferation analysis. Cell proliferation was assessed using the MTT assay. SGC7901 cells (transfected and untreated) were seeded in 96-well microplates at a density of 2,000 cells per well in RPMI-1640 medium containing 10% FBS. Cells were incubated for 0, 24, 48 and 72 h. A total of 20 μ l MTT substrate (5 mg/ml in RPMI-1640) was added to each well and the plates were incubated for another 4 h at 37°C. The reaction was terminated by adding 150 μ l dimethyl sulfoxide. The optical density (OD) was detected using the Stat Fax® 2100 multi-well plate reader (Awareness Technology, Inc., Palm City, FL, USA) by measuring absorbance at 570 nm with background subtraction of 690 nm. Each assay was performed in triplicate and repeated three times. Growth curves were constructed with OD₅₇₀ on the vertical axis and culture time on the horizontal axis.

Colony formation assay. Anchorage-dependent cell growth was determined by analyzing the formation of colonies on the plates. Briefly, 100 cells were plated on six-well plates and incubated for two weeks to allow for colony formation. The colonies were then fixed with 70% ethanol, stained with Giemsa solution and counted. The assay was performed in triplicate.

Flow cytometric analysis of the cell cycle. For flow cytometric analysis of the cell cycle, the cells were seeded and maintained on 6 cm-diameter plates in RPMI-1640 containing 10% FBS overnight. The cells were synchronized in a serum-free medium for 24 h and then cultured again in RPMI-1640 containing 10% FBS. After 24 h of incubation, a total of 1x10⁷ cells was harvested and fixed in 70% cold ethanol for 45 min. Following repeated washings, the cells were stained with a solution containing 40 μ g/ml propidium iodide and 100 μ g/ml RNase A. The samples were immediately analyzed by a FAC-Scan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). The distribution of the cell cycle was determined using CellQuest™ Pro software (BD Biosciences, Franklin Lakes, NJ, USA) and ModFit LT™ software (Verity Software House, Topsham, ME, USA). Three independent experiments were performed.

Tumorigenicity in nude mice. A total of 5x10⁶ logarithmically growing cells were resuspended in 0.2 ml RPMI-1640 medium and injected into the right oter of the 4-5 week old female Balb/c-nu/nu mice. Each experimental group consisted of four mice. Tumor growth was measured every three days after injection using calipers. All mice were sacrificed on day 21 after injection. The sizes of the tumors were calculated

Figure 1. Stable downregulation of 14-3-3ε protein expression. The image is a representative of three separate experiments. Parental, parental SGC7901 cells; vector, cells transfected with scramble shRNA; shRNA1/2, cells transfected with 14-3-3ε shRNA. β-actin served as an internal control. The expression levels of 14-3-3ε protein were significantly lower in the shRNA1 and shRNA2 groups than in the control vector and parental groups. *P<0.05 vs. shRNA1 group, shRNA2 group and parental group; #P>0.05 vs. vector group and parental group; @P>0.05 vs. shRNA1 group and shRNA2 group. sh, short hairpin.

Figure 2. Effect of 14-3-3ε downregulation on cell proliferation *in vitro*. Cell proliferation was evaluated by the absorbance value at 570 nm and cell growth curves were plotted using culture time on the horizontal axis and OD (570 nm) on the vertical axis. Error bars correspond to the mean ± standard error of the mean. The graph shows that cell proliferation is markedly reduced when 14-3-3ε is downregulated in SGC7901 cells. *P<0.05 vs. shRNA1 group, shRNA2 group and parental group; #P>0.05 vs. vector group and parental group; @P>0.05 vs. shRNA1 group and shRNA2 group. sh, short hairpin; OD, optical density.

according to the following formula: Length x width²/2, where length was the longest diameter and width was the shortest diameter of each tumor.

Statistical analyses. The data were presented as the mean ± standard deviation or the mean ± standard error of the mean. The statistical analyses were performed using SPSS software, version 19.0 (IBM, Armonk, NY, USA). Comparisons among all groups were analyzed by one-way analysis of variance and P<0.05 was considered to indicate a statistically significant difference.

Figure 3. Effect of 14-3-3ε downregulation on plate colony formation. Stable transfectants (1×10²) were seeded onto six-well plates and cultured for 14 days. The number of foci was counted. Vertical bars show the mean ± standard deviation of three separate experiments. The results indicate that 14-3-3ε downregulation resulted in inhibition of the colony formation rate in the SGC7901 cell line. *P<0.05 vs. shRNA1 group, shRNA2 group and parental group; #P>0.05 vs. vector group and parental group; @P>0.05 vs. shRNA1 group and shRNA2 group. sh, short hairpin.

Results

Stable downregulation of 14-3-3ε in the SGC7901 cell line. SGC7901 human gastric cancer cells were transfected with 14-3-3ε shRNA and control shRNA, respectively. Following puromycin selection, western blot analysis was performed to determine the knockdown efficiency of 14-3-3ε. As shown in Fig. 1, the expression levels of 14-3-3ε protein were significantly reduced in the shRNA1 and shRNA2 groups in comparison with the parental SGC7901 and vector groups (cells transfected with control shRNA). The experiment was repeated three times. The three independent experiments confirmed that 14-3-3ε protein expression was successfully downregulated in the shRNA1 and shRNA2 groups, but no significant differences in 14-3-3ε expression levels were detected between the cells transfected with the control vector and the parental group.

14-3-3ε downregulation inhibits cell proliferation and colony formation *in vitro*. To determine the impact of 14-3-3ε on gastric cancer cell proliferation, parental SGC7901 cells and the cells from the variant groups were seeded onto 96-well culture plates and growth was analyzed every day by MTT assay. As revealed by the growth curves in Fig. 2, the vector cells showed a similar growth rate to parental SGC7901 cells, whereas the shRNA1 and shRNA2 cells exhibited a significantly lower growth rate compared with the parental SGC7901 cells (P<0.05).

To investigate whether the levels of 14-3-3ε expression affect the colony formation ability *in vitro*, a colony formation assay was conducted to detect cell growth viability in shRNA1-, shRNA2- and vector-transfected as well as parental SGC7901 cells. The colony formation capacity was estimated at 14 days after transduction. As shown in Fig. 3, the colony formation rates of shRNA1 cells and shRNA2 cells were significantly reduced in comparison with those of the parental SGC7901 cells (P<0.05). These results confirm the anti-proliferative role of 14-3-3ε in gastric cancer cells.

Figure 4. Effect of 14-3-3 ϵ downregulation on cell cycle distribution. (A) Cell cycle distribution images shown are representative of three independent experiments. (B) Percentages of cells in the G0/G1, S or G2 phases of the cell cycle (mean $\pm$ standard deviation) from three independent experiments. The fluorescence-activated cell sorting analysis revealed that 14-3-3 ϵ downregulation resulted in G0/G1 phase arrest in SGC7901 cells. * P <0.05 vs. shRNA1 group, shRNA2 group and parental group; # P >0.05 vs. vector group and parental group; @ P >0.05 vs. shRNA1 group and shRNA2 group. sh, short hairpin.

14-3-3 ϵ downregulation triggers G0/G1 arrest in the SGC7901 cell line. To uncover the mechanism underlying the growth suppression effect of 14-3-3 ϵ on SGC7901 cells, the cell cycle distribution was analyzed by fluorescence-activated cell sorting analysis. As shown in Fig. 4, 14-3-3 ϵ knockdown induced cell cycle arrest in G0/G1 phase in the SGC7901 cell line. As compared with the parental SGC7901 cells, the percentage of cells in G0/G1 phase in the shRNA1 and shRNA2 groups was significantly increased by 13.06 and 17.81% (P <0.05), and the percentage of cells in S phase was significantly reduced by 6.94 and 5.85% (P <0.05), respectively. However, no significant differences in cell cycle distribution were observed between the parental SGC7901 cells and the cells transfected with control shRNA. This suggests that 14-3-3 ϵ downregulation induced G0/G1 arrest in SGC7901 cells.

14-3-3 ϵ downregulation inhibits tumor growth of gastric cancer cells *in vivo*. To further confirm the effects of 14-3-3 ϵ on the tumor growth of gastric cancer, shRNA1-, shRNA2- and vector-transfected as well as parental SGC7901 cells were inoculated into female nude mice. Three weeks after injection, the mice were sacrificed and the tumors were excised. As shown in Fig. 5, the velocities of tumor growth in the shRNA1 and shRNA2 groups were significantly

slower than those of the parental SGC7901 or vector groups (P <0.05). These results indicated that downregulation of 14-3-3 ϵ expression resulted in inhibited tumor growth *in vivo*.

Inhibition of 14-3-3 ϵ suppresses cyclin E expression and induces p27^{kip1} expression in SGC7901 cells. Cyclin E is an important cell cycle regulator, which forms a complex with cyclin-dependent kinase (CDK) 2 and facilitates cell cycle progression from G1 to S phase. P27^{kip1}, known as a CDK inhibitor, mainly inhibits the activity of cell cycle complexes, including cyclin E-CDK2, which results in G1 phase arrest (23). Western blot analysis was performed to detect the protein expression levels of these cell cycle regulators. As indicated in Fig. 6, the expression of p27^{kip1} was upregulated and the expression of cyclin E was downregulated in the shRNA1 and shRNA2 groups (P <0.05). The results demonstrated that 14-3-3 ϵ may have been involved in regulating the expression of cyclins binding to CDKs and the expression of CDK inhibitors in the SGC7901 gastric cancer cell line.

Discussion

Increasing evidence suggests that 14-3-3 ϵ is critical in carcinogenesis and cancer progression. Altered 14-3-3 ϵ expression

Figure 5. Effect of 14-3-3 ϵ downregulation on xenografts *in vivo*. Parental, control vector and 14-3-3 ϵ -shRNA-transfected SGC7901 cells were injected subcutaneously into nude mice. Tumor volume was measured at seven-day intervals for 21 days and calculated according to the formula: Volume = length(mm) $\times$ width²/2. Growth curves showed that tumor volumes in the shRNA1 and shRNA2 groups increased at markedly lower rates than those in the parental and control vector groups. Values show mean $\pm$ standard deviation. * P <0.05 vs. shRNA1 group, shRNA2 group and parental group; # P >0.05 vs. vector group and parental group; @ P >0.05 vs. shRNA1 group and shRNA2 group. sh, short hairpin.

Figure 6. Protein expression levels of cell cycle-associated molecules. Total cellular protein was extracted and the expression levels of specific proteins were determined by western blot analysis using antibodies against cyclin E and p27. β -actin served as a loading control. The image presents one out of three separate experiments. The protein expression levels of cyclin E were significantly lower in the shRNA1 and shRNA2 groups than in the control vector and parental groups, while the protein expression levels of p27 were higher in the shRNA1 and shRNA2 groups than in the control vector and parental groups. * P <0.05 vs. shRNA1 group, shRNA2 group and parental group; # P >0.05 vs. vector group and parental group; @ P >0.05 vs. shRNA1 group and shRNA2 group. sh, short hairpin.

levels have been implicated in the initiation and development of different types of tumors. Previous studies have shown that the role of 14-3-3 ϵ in the progression of specific tumors is controversial. However, it has been demonstrated that 14-3-3 ϵ expression is upregulated and functions as an oncogene in a number of tumors, including breast and hepatic cellular cancer, vulvar squamous cell carcinoma, follicular and papillary thyroid tumors and meningioma (13-17). For example, Ko *et al* (17) demonstrated that overexpression of 14-3-3 ϵ in primary hepatic cellular cancer tissues conveys a high risk of extrahepatic metastasis and shortened survival rate. In addition, 14-3-3 ϵ has been shown to act as an antioncogene in certain instances. Che *et al* (24) revealed that reduced expression levels of 14-3-3 ϵ contributed to laryngeal squamous cell carcinoma (LSCC) development and that 14-3-3 ϵ was able to promote apoptosis and inhibit the invasiveness of LSCC. This discrepancy in response to 14-3-3 ϵ is suggestive of a specificity for different cell types. To date, little is known regarding the role of 14-3-3 ϵ in gastric cancer.

Preliminary results performed by our group using immunohistochemical staining revealed that the protein expression levels of 14-3-3 ϵ in gastric cancer tissues were significantly higher than those of paired tumor tissues. The present study attempted to further demonstrate the involvement of 14-3-3 ϵ in cell growth by using RNAi. MTT and colony formation assays showed that the proliferation of gastric cancer cells was significantly inhibited in cells in which 14-3-3 ϵ was downregulated. Furthermore, tumorigenicity in xenografts was reduced upon 14-3-3 ϵ downregulation. The above results indicated that 14-3-3 ϵ may act as an oncogene in gastric cancer. However, downregulation of 14-3-3 ϵ reduced but did not completely halt cell proliferation, indicating that, although 14-3-3 ϵ was clearly involved in gastric cancer cell proliferation, other proteins were also involved in this process. In addition, a component of the tumor-promotion activity of 14-3-3 ϵ was demonstrated to proceed via the inhibition of cell cycle progression as indicated by increased accumulation of cancer cells in the G1 phase and a corresponding reduction of cells in the S phase of the cell cycle in the SGC7901 gastric cancer cell line.

The exact molecular mechanism of how 14-3-3 ϵ downregulation stimulates G1-S arrest remains to be elucidated. One notable study suggested that 14-3-3 ϵ regulates compact ventricular myocardium growth by modulating the cardiomyocyte cell cycle via cyclin E and p27^{kip1} (25). In the present study, a possible correlation between 14-3-3 ϵ and cyclin E/p27^{kip1} protein expression was investigated *in vitro*. The study revealed that the expression levels of cyclin E were reduced, whereas the expression levels of p27^{kip1} were increased upon 14-3-3 ϵ knockdown in SGC7901 cells. It is known that p27^{kip1} is a CDK inhibitor acting on cyclin E-CDK2, which promotes G1/S phase transition (26,27). Therefore, changes in the expression levels of cyclin E and p27^{kip1} may contribute to the effect of 14-3-3 ϵ on gastric cancer cell proliferation and G1/S checkpoint control. Of note, 14-3-3 ϵ in association with DP-3 has also been reported to upregulate the transcription of cyclin E (28). 14-3-3 ϵ was originally considered to regulate signal transduction by modulating protein-protein interactions in the cytoplasm (29). A mechanism by which 14-3-3 ϵ may promote the progression of the cell cycle may be through facilitating the localization of p27^{kip1} to the cytoplasm (30,31),

where phosphorylated p27^{kip1} is degraded by proteases following ubiquitination (32). In future studies, the analysis of 14-3-3 ϵ interactions with protein-binding partners may reveal the mechanisms by which 14-3-3 ϵ modulates the expression levels of different cell cycle-associated proteins.

The findings of the present study are contradictory to those of Leal *et al* (33), who demonstrated that 14-3-3 ϵ expression levels are reduced in gastric cancer, comparing 14-3-3 ϵ protein expression levels in 20 pairs of gastric cancer samples with corresponding non-neoplastic gastric tissues using western blot analysis. The insufficient sample size and the lack of further investigation *in vitro* and *in vivo* may be responsible for this difference. In addition, the role of 14-3-3 ϵ in other gastric cancer cell lines and the manner in which cyclin E and p27^{kip1} are regulated require further investigation.

In conclusion, the present study demonstrated that knock-down of 14-3-3 ϵ inhibited the proliferation of gastric cancer cells, partially through downregulation of cyclin E and upregulation of p27^{kip1}. This provides novel insight into the function of 14-3-3 ϵ and suggests that downregulation of 14-3-3 ϵ by RNAi may be another possible approach in the management of human gastric cancer.

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