

Overexpression of SIRT4 inhibits the proliferation of gastric cancer cells through cell cycle arrest

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Abstract. The sirtuins (SIRT) are a family of nicotinamide-adenine dinucleotide (NAD)⁺-dependent protein deacetylases. SIRT4 is a mitochondrial NAD⁺-dependent adenosine diphosphate-ribosyltransferase. Recent studies demonstrated that SIRT4 can regulate glutamine metabolism and thus act as a tumor suppressor. However, the association of SIRT4 with gastric cancer remains unknown. The present study investigated the potential role of SIRT4 in the proliferation of human gastric cancer cells. Gastric cancer cell lines (SGC-7901 and MNK45) overexpressing SIRT4 were established by lentiviral infection. The effect of overexpression of SIRT4 in gastric cancer was evaluated by determining the cell viability, proliferation activity and colony-forming ability of gastric cancer cells *in vitro*. Furthermore, the cell cycle profiles of SGC-7901 and MNK45 cells overexpressing SIRT4 were evaluated to provide insights into potential underlying molecular mechanisms. Overexpression of SIRT4 significantly inhibited the proliferation and colony-forming ability of the gastric cancer cells *in vitro*. Furthermore, overexpression of SIRT4 induced G1 cell cycle arrest via suppression of phosphorylated extracellular signal-regulated kinase, cyclin D and cyclin E. In conclusion, the results of the present study indicated that SIRT4 may function as a tumor suppressor in gastric cancer by regulating cell proliferation, therefore SIRT4 may be a potential therapeutic target against this disease.

Introduction

Gastric cancer is the third leading cause of cancer-associated mortality globally, accounting for 723,000 mortalities or 8.8% of all cancer-associated mortalities in 2012 (1). Tubular adenocarcinoma is the most predominant histologic type of gastric cancer, followed by the papillary and mucinous types (1). The incidence rate of gastric cancer has substantially declined over the past few decades; however, the median survival rate remains poor (2). Furthermore, gastric cancer is a heterogeneous disease with complex etiology and pathogenesis, involving a variety of risk factors, including dietary, infectious, genetic and epigenetic alterations (2).

Over the past few decades, numerous putative candidate genes and signaling pathways have been reported to serve a crucial role in the development and progression of gastric cancer. These include the tumor protein p53, phosphoinositide-3-kinase, AT-rich interactive domain-containing protein 1A, Wnt/ β , transforming growth factor β and Notch signaling pathways (3). Therefore, understanding the underlying molecular mechanisms of gastric cancer may provide novel insights into the pathogenesis of gastric cancer and help identify novel potential biomarkers and therapeutic targets for treatment.

The sirtuins (SIRT) are a family of nicotinamide-adenine dinucleotide (NAD)⁺-dependent protein deacetylases. Humans encode seven SIRT orthologues, SIRT1-SIRT7, which exhibit varying intracellular distribution (4). These SIRTs are known to serve an important role in stress resistance, genome stability, energy metabolism and aging (4). Previously, a number of studies have indicated the role of SIRT in tumor development, survival and tumor metabolism (5). SIRT4 utilizes NAD⁺ for adenosine diphosphate-ribosylation to downregulate the activity of glutamate dehydrogenase and suppress insulin secretion from pancreatic β -cells (6). Previous studies demonstrated that mitochondrial SIRT4 may function as a tumor suppressor by regulating the metabolism of glutamine, which indicates that it may exhibit a therapeutic potential in cancer (7,8). Additionally, previous studies have identified an association of SIRT4 expression in colon and esophageal cancer with a reduction in adverse outcomes associated with these tumors (9-11). Furthermore, our previous study demonstrated that a reduced expression level of SIRT4 protein is associated with gastric cancer (12).

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However, the function of SIRT4 in gastric cancer cells remains unknown.

The present study reported that overexpression of SIRT4 inhibits the proliferation of gastric cancer cells via G1 cell cycle arrest by inhibiting the expression of cyclin D, cyclin E and phosphorylated extracellular signal-regulated kinase (p-ERK). In summary, the results of the present study demonstrate the tumor suppressive function of SIRT4 in gastric cancer and indicate its potential as a therapeutic target for this disease.

Materials and methods

Cell lines and culture conditions. Human gastric cancer cell lines SGC-7901 and MNK45 were obtained from the Shanghai Institute of Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc.) and penicillin (100 U/ml) /streptomycin (0.1 mg/ml) at 37°C and 5% CO₂.

Construction and transfection of the SIRT4 overexpression vector. pHBLV-CMVIE-ZsGreen-T2A-Puro, the lentivirus vector that induces overexpression of SIRT4 and the empty vector were purchased from Hanbio Biotechnology Co., Ltd. (Shanghai, China). The final titer of the lentivirus and the negative control virus was 2x10⁸ PFU/ml. The transfection MOI was 10. Stable overexpression of SIRT4 was achieved by infecting SGC-7901 and MNK45 cells with lentiviruses for 72 h followed by culturing in high glucose Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) with 2 µg/ml puromycin (Beyotime Institute of Biotechnology, Haimen, China) at 37°C and 5% CO₂ for 2 weeks.

Cell proliferation assay. To observe cell proliferation activity, cells were seeded in 96-well plates at a density of 1,000 cells/well. Following incubation for 12, 36, 60, 84 or 108 h, detection of each well was enabled by adding 10 µl Cell Counting Kit-8 reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) and the absorbance was read by a spectrophotometer at 450 nm following culturing in the CO₂ incubator at 37°C for 2 h.

Colony formation assay. Logarithmic growth phase cells were plated at a density of 100 cells/well in six-well plates and cultured for 2 weeks. Cells were then fixed in 100% methanol for 15 min at 37°C and stained using Giemsa stain at 37°C for 30 min to permit direct counting of the number of colonies formed with the naked eye.

Flow cytometry analysis of cell cycle. The cell cycle assay was performed using flow cytometry. Briefly, cells (1x10⁶) were washed twice with ice-cold PBS, treated with trypsin and subsequently washed with PBS containing 3% fetal bovine serum. Prior to analysis, cells were stained using a propidium iodide cell cycle detection kit (BD Biosciences, Franklin Lakes, NJ, USA) at room temperature for 30 min. Analysis was performed using a FACScan flow cytometer (BD Biosciences).

The cell cycle results were analyzed using the ModFit analysis software program (version 4.0; Verity Software House, Inc., Topsham, ME, USA).

Western blot analysis. Cells were lysed using ice-cold radio-immunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) supplemented with protease. The cellular lysates were collected and the protein content was determined using a Bicinchoninic Acid assay kit (Beyotime Institute of Biotechnology). For western blot analysis, 40 µg of protein was resolved on 12% SDS-PAGE and then transblotted to methanol-activated polyvinylidene difluoride membranes. The resulting blots were blocked with 10% fat-free milk for 1 h in TBS with 0.1% Tween and incubated with the appropriate primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:1,000; catalog no. ab97200; Abcam) at room temperature for 30 min. Finally, protein bands were detected using an enhancement chemiluminescent substrate (EMD Millipore, Billerica, MA, USA) and quantification was performed using ImageJ software (version 2.1.4.7; National Institutes of Health, Bethesda, MD, USA). The following primary antibodies were used for this western blot analysis: Rabbit anti-human SIRT4 polyclonal antibody (1:1,000; catalog no. HPA029691; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), rabbit anti-human cyclin D monoclonal antibody (1:1,000; catalog no. 60816-1-IG, ProteinTech Group, Inc., Chicago, IL, USA), rabbit anti-human cyclin E monoclonal antibody (1:1,000; catalog no. Ab33911; Abcam, Cambridge, MA, USA), rabbit anti-human ERK polyclonal antibody (1:1,000; catalog no. 9102; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-human p-ERK polyclonal antibody (1:1,000; catalog no. 4370; Cell Signaling Technology, Inc.), rabbit anti-human β-actin polyclonal antibody (1:1,000; catalog no. ab11971; Abcam) and rabbit anti-human GAPDH polyclonal antibody (1:1,000; catalog no. AB-P-R 001; Hangzhou Goodhere Biotechnology Co., Ltd., Hangzhou, China).

Statistical analysis. Statistical analysis was performed using the statistical software program SPSS version 20.0 (IBM Corp., Armonk, NY, USA). All *in vitro* experiments were performed in triplicate. Data from three or more independent experiments are presented as the mean ± standard deviation. Student's t-test was performed to determine the differences between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Overexpression of SIRT4 inhibits proliferation of human gastric cancer cells. Stable strains of human gastric cancer cell lines SGC-7901 and MNK45 were constructed by lentiviral infection and overexpression of SIRT4 was confirmed by western blot analysis (Fig. 1A). A significant inhibition in the proliferation rates of SGC-7901 and MNK45 cells was observed following SIRT4 overexpression (Fig. 1B and C). Furthermore, a colony formation assay revealed that SIRT4 overexpression significantly reduced the number of colonies formed by SGC-7901 and MNK45 cells *in vitro* (Fig. 1D).

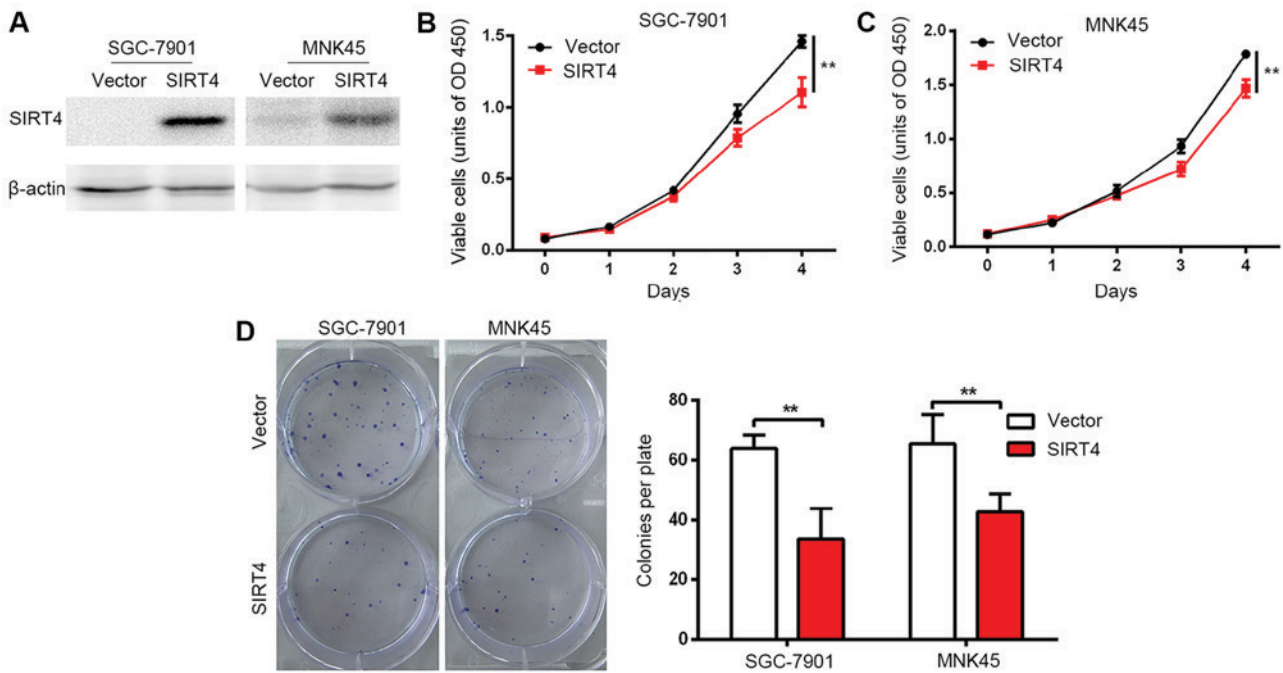


Figure 1. Overexpression of SIRT4 inhibits the proliferation of gastric cancer cells. (A) SGC-7901 and MNK45 cells were transfected with a negative control vector and a SIRT4 expressing lentiviral vector. After 2 weeks, expression of SIRT4 was detected by western blot analysis and β -actin was used as an internal control. Proliferation activity curves were generated for (B) SGC-7901 and (C) MNK45 cells transfected with the SIRT4-overexpression vector or empty control vector. Cells were seeded at a density of 1,000 cells/well in 96-well plates and the cell viability was determined by cell counting kit-8 assay every 24 h for 4 days. (D) A representative experimental image following SIRT4 transfection of SGC-7901 and MNK45 cells to observe colony formation. Cells were cultured for 14 days and stained with Giemsa. Colony formation was determined by counting the number of colonies per plate. Data are presented as the mean \pm standard deviation (n=3). **P<0.01. OD, optical density; SIRT4, sirtuin 4.

These results indicated that SIRT4 inhibits the cell growth and proliferation rates of gastric cancer cells *in vitro*.

Overexpression of SIRT4 induces G1 cell cycle arrest in gastric cancer cells. To further determine whether SIRT4 inhibits the proliferation of human gastric cancer cells by arresting the cell cycle, the cell cycle profiles of SIRT4-overexpressing SGC-7901 and MNK45 cells were analyzed using flow cytometry and propidium iodide staining. Overexpression of SIRT4 significantly increased the proportion of cells in the in G1 phase and reduced the number of cells in the S phase of the cell cycle, compared with the controls (Fig. 2). Furthermore, overexpression of SIRT4 significantly increased the proportion of SGC-7901 cells in the G2 phase (Fig. 2B). Cell growth inhibition by SIRT4 overexpression was associated with significant cell cycle arrest at the G1 phase, which indicates that overexpression of SIRT4 suppresses cell proliferation by G1 cell cycle arrest and induces specific inhibition of cell cycle progression. By contrast, overexpression of SIRT4 did not affect apoptosis of gastric cancer cells (data not shown).

SIRT4 regulates the expression of cell cycle G1-associated proteins. To validate the results of flow cytometry, the expression of G1 cell cycle regulatory proteins were detected by western blot analysis. It was identified that SIRT4 significantly inhibited the expression of cyclin D and cyclin E (Fig. 3). Additionally, SIRT4 overexpression was associated with a significant decrease in the expression level of p-ERK, which indicates a reduced level of activated ERK. These results

indicated that SIRT4-induced G1 cell cycle arrest is associated with the suppression of ERK, cyclin D and cyclin E.

Discussion

Numerous SIRT family members serve a role in tumor development and different SIRTs are localized in different subcellular compartments, and can thus modulate different targets in the cell (13). For example, SIRT1 is highly expressed in gastric (14), colon (15), prostate (16) and skin cancer (17), which indicates that it serves a role in promoting tumor formation in these tissues. By contrast, other studies demonstrated that SIRT1 expression is reduced in breast cancer (18) and its expression in a mouse model has been revealed to prevent the formation of intestinal tumors (19). Furthermore, similar observations have been reported for SIRT2, which has been identified to be downregulated in breast cancer (20), glioma (21) and skin cancer (22), but overexpressed in acute myeloid leukemia (23) and prostate cancer (24). Therefore, it remains unclear whether the observations made for one tumor type can be extrapolated to conclude the role of SIRTs in other tumor types.

A limited number of studies investigated the biological functions and significance of SIRT4 in tumors. Jeong *et al* (7) demonstrated that SIRT4 inhibits the formation of tumor by suppressing glutamine metabolism. Overexpression of SIRT4 inhibited the growth of HeLa cells and SIRT4-knockout MEF cells in nude mice reduced their ability to form large tumors. Furthermore, SIRT4-knockout mice spontaneously produced lung, liver, breast and lymphoma cancer. Csibi *et al* (8) indicated that overexpression of SIRT4 reduces the growth of the human

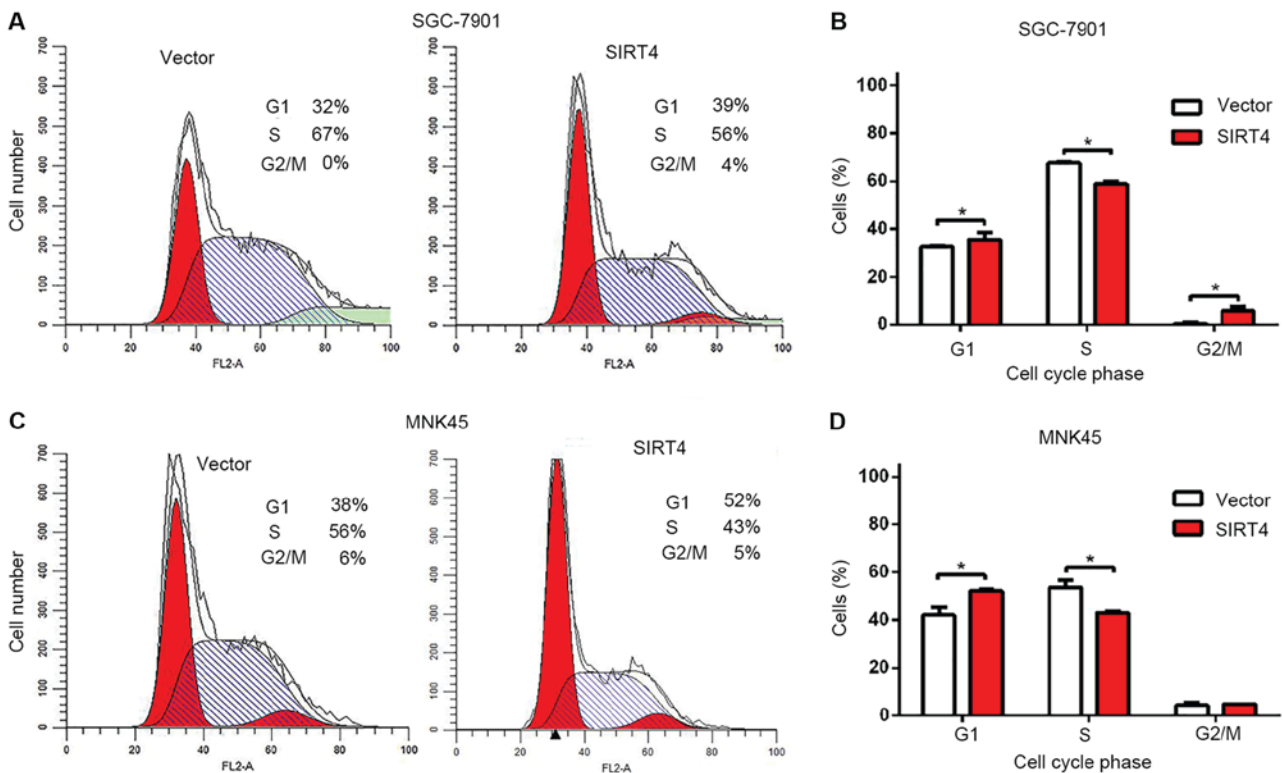


Figure 2. Overexpression of SIRT4 inhibits the cell cycle in gastric cancer cells. (A) The percentage of SGC-7901 cells transfected with SIRT4-overexpression vector or empty control vector at different phases of the cell cycle were detected using a PI cell cycle detection kit. The results were analyzed using Modfit software. (B) The statistical results of the cycle distribution of SGC-7901 cells. (C) The percentage of MNK45 cells transfected with SIRT4-overexpression vector or empty control vector at different phases of the cell cycle were detected using a PI cell cycle detection kit. The results were analyzed using Modfit software. (D) The statistical results of the cycle distribution of MNK45 cells. Data are presented as the mean \pm stand deviation. * $P < 0.05$. PI, propidium iodide; SIRT4, sirtuin 4.

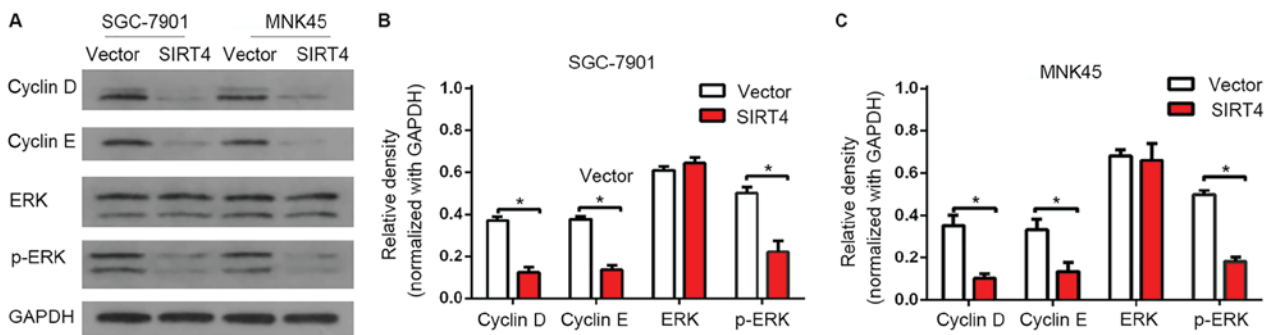


Figure 3. SIRT4 regulates the expression of cell cycle G1-associated proteins. (A) The expression levels of cyclin D, cyclin E, ERK, p-ERK and GAPDH were detected by western blot analysis. (B) The relative expression levels of cyclin D, cyclin E, ERK and p-ERK proteins in SGC-7901 cells were quantified according to the expression level of GAPDH. (C) The relative expression levels of cyclin D, cyclin E, ERK and p-ERK proteins in MNK45 cells were quantified according to the expression level of GAPDH. Expression levels were analyzed and quantified using ImageJ software. Data are representative of three independent experiments and are expressed as the mean \pm standard deviation. * $P < 0.05$. p-ERK, phosphorylated extracellular signal-regulated kinase; SIRT4, sirtuin 4.

colon cancer cell line DLD-1 and the human prostate cancer cell line DU145. Additionally, Jeong *et al* (25) identified that SIRT4 inhibits the growth of Myc-induced B cell lymphoma.

Our previous study and another study revealed that SIRT4 expression in colon and esophageal cancer is associated with a reduction in adverse outcomes (9,10). Furthermore, we previously reported that SIRT4 expression was associated with pathological parameters in gastric cancer, including pathological stage, T stage and UICC stage (12). The present study revealed that SIRT4 inhibits the proliferation of gastric cancer

cells *in vitro*. The experimental results indicate that SIRT4 serves a crucial role as a tumor suppressor in gastric cancer.

To further analyze the underlying mechanism involved in the inhibition of cell proliferation in gastric cancer cells following overexpression of SIRT4, the cell cycle distribution was analyzed by flow cytometry. Overexpression of SIRT4 arrested the cell cycle at the G1 phase in SGC-7901 and MNK45 cells. Additionally, it was identified that overexpression of SIRT4 significantly reduced the expression of cyclin D, cyclin E and p-ERK in gastric cancer cells. A previous study

indicated that increased expression of cyclin D in cancer cells results in uncontrolled cell growth (26). ERK has been reported to regulate the G1 cell cycle phase via modulation of cyclin D (27). Cyclin E has also been reported to exhibit a critical role in regulating the G1 to S phase transition (28,29). The observations of the present study indicated that SIRT4-induced G1 cell cycle arrest is associated with the suppression of ERK, cyclin D and cyclin E.

To the best of our knowledge, no previous study has reported the function of SIRT4 in gastric cancer cells. The present *in vitro* study demonstrated that SIRT4 overexpression could significantly inhibit the cell proliferation of gastric cancer cells and arrest the cell cycle by suppressing ERK, cyclin D and cyclin E. In summary, the results of the present study highlight the tumor suppressive role of SIRT4 in gastric cancer and indicate its potential as a therapeutic target for this disease.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YH, JL, YL and XC performed the experiments. GZ and GH performed the statistical analysis and wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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