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Original Research Article

Shikonin suppresses the proliferation and colony formation of gastric cancer cells by regulating miR-96/SOCS4 pathway

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Abstract

Purpose: *To investigate whether shikonin is able to inhibit cell proliferation and colony formation in gastric cancer (GC) cells and to elucidate the molecular mechanism.*
Methods: *Gastric cancer (GC) cell line SGC-7901 was used. The effects of shikonin on SGC-7901 cells were evaluated using Cell Counting Kit-8 (CCK-8) and soft-agar colony formation assays, respectively. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was employed to measure miR-96 expression levels. The regulatory effect of miR-96 on SOCS4 was determined by luciferase activity assay, while the effect of shikonin and miR-96 overexpression on proliferating cell nuclear antigen (PCNA), cyclin D1, suppressor of cytokine signaling 4 (SOCS4), and JAK/STAT pathway-related protein expression levels were analyzed by western blots.*
Results: *The results show that shikonin dose-dependently suppressed the proliferation and colony formation of SGC-7901 cells. Western blot analysis revealed that PCNA and cyclin D1 were downregulated by shikonin treatment. Luciferase activity assay demonstrated that miR-96 is directly bound to SOCS4. Further results showed that miR-96 mimics reversed the effects of shikonin on SOCS4 and JAK/STAT pathway-related protein expression levels.*
Conclusion: *Shikonin suppresses proliferation and colony formation by regulating miR-96/SOCS4 pathway in SGC-7901 cells, providing a potential therapeutic target for GC.*

Keywords: *Shikonin, Gastric cancer, MiR-96, SOCS4*

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INTRODUCTION

Gastric cancer (GC) is one of the most common adenocarcinomas worldwide and it ranks second in cancer-related mortality [1-3]. Despite the declined incidence of GC, little is known about the pathogenesis of GC, which may lead to poor prognosis and high lethality [2,3]. Therefore, the identification of new compounds for better prognosis of GC is still a demanding issue.

Shikonin is a natural naphthoquinone of *Zicao* that derived from the dried root of the herb *Lithospermum erythrorhizon* [4-12]. Shikonin is

effective in various diseases and exerts abilities such as anti-inflammation, anti-infection, and wound healing [4-12]. Recent studies have described the anti-tumorigenic bioactivities of shikonin [4-12]. Shikonin can cause premature senescence of lung cancer cells [5]and induce apoptosis of hepatocellular carcinoma [6]. Its derivatives exhibit anti-leukemic activity [8]. Shikonin promotes the generation of reactive oxygen species (ROS), which induces the apoptosis of GC cells [9]. It suppresses cell invasion and migration by inhibition of Toll-like receptor 2/nuclear factor-kappa B pathway [11]. The mammalian target of rapamycin (mTOR) signaling pathway has been a target for potential therapeutic strategies in various cancer cells [13-16]. Phosphatase and tensin homolog (*PTEN*), a tumor suppressor gene, is a primary negative regulator of the phosphoinositide-3 kinase (PI3K)/protein kinase B (AKT)/mTOR signaling pathway [12,17]. A recent study has demonstrated that shikonin suppresses proliferation and promotes apoptosis by modulating PTEN/AKT/mTOR signaling through downregulation of microRNA (miRNA) miR-106b expression in human endometrioid cancer cells [12]. These findings demonstrate that various signaling pathways are regulated by shikonin.

Small non-coding RNA molecules known as miRNAs, are important for RNA silencing and gene regulation at the post-transcriptional level [18,19]. During the past decades, compelling reports demonstrated that miRNAs participate in almost all aspects of cancer biology such as metastasis, apoptosis, and proliferation, suggesting that miRNAs are also potential biomarkers for cancer diagnosis and prognosis [18-22]. However, the mechanisms of miRNA in GC are still unclear, and further investigation is necessary to elucidate the potential applications of miRNAs.

EXPERIMENTAL

Human cell lines and reagents

Human SGC-7901 and 293T cell line were from the Chinese Academy of Sciences, Shanghai Cell Bank (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM) medium, fetal bovine serum (FBS), penicillin, streptomycin, phosphate-buffered saline (PBS), and trypsin-EDTA (0.25 %) were purchased from Gibco Life Technologies (Shanghai, CN, China). Shikonin and the Cell Counting Kit-8 (CCK-8) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), proliferating cell nuclear antigen (PCNA), cyclin D1, proto-oncogene serine/threonine-protein kinase (PIM), and transporter associated with antigen processing 1 (TAP1) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against suppressor of cytokine signaling 4 (SOCS4) were supplied by Sigma-Aldrich. Antibodies against signal transducer and activator of transcription 3 (STAT3) and p-STAT3 were provided by Thermo Fisher Scientific (Invitrogen, San Jose, CA, USA). Secondary antibodies, horseradish peroxidase (HRP)-conjugated anti-mouse IgG and anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. The information and preparations of the primary antibodies against the specific proteins are as follows: mouse monoclonal GAPDH antibody (sc-47724, dilution 1:500 in 1 × TBST), mouse monoclonal PCNA antibody (sc-56, dilution 1:500 in 1 × TBST), mouse monoclonal PIM antibody (sc-13513, dilution 1:500 in 1 × TBST), mouse monoclonal TAP1 antibody (sc-6246, dilution 1:500 in 1 × TBST), mouse polyclonal SOCS4 antibody (SAB1408273, dilution 1:500 in 1 × TBST), mouse monoclonal STAT3 antibody (AB_2533031, dilution 1:500 in 1 × TBST), rabbit polyclonal p-STAT3 antibody (AB_2533643, dilution 1:500 in 1% BSA-TBST), the secondary antibodies; anti-mouse IgG -HRP (sc-2005, dilution 1:5,000 in 1 × TBST), and anti-rabbit IgG-HRP (sc-2357,dilution 1:5,000 in 1 × TBST).

Cell culture

Human SGC-7901 cells and human 293T cells were maintained in DMEM medium supplemented with penicillin (50 U/mL), FBS (10 %) and streptomycin (50 µg/mL) in 5 % CO_2 at 37 °C.

Soft-agar colony formation assay

DMEM medium (5 mL) containing 0.75 % agar was used for the base agar layer. Cells (3×10^4 cells/mL) were resuspended into 3 mL DMEM medium containing 0.36 % agar for the top layer. The cells were cultured in 5 % CO_2 at 37 °C for 3 weeks. Then, the colonies were stained with crystal violet (0.04 %) in PBS containing ethanol (2 %). The stained colonies were imaged using viewPIX 700 – Colorimetric Gel Scanner.

CCK-8 assay

The SGC-7901 cells were diluted to 8×10^4 cells/mL, at 100 µL/well in a 96-well plate. Shikonin was added and incubated. CCK-8 solution (10 µL per well) was added. The plate was incubated at 37°C for 2 h and the

absorbance at A450 nm was measured using a microplate reader.

Western blot analysis

Cells (8 × 10^4 cells/mL) were detached and harvested with centrifugation. The cells were washed twice with PBS, and resuspended in radioimmunoprecipitation assay buffer (RIPA buffer) supplemented with protease and phosphatase inhibitors. The supernatants were collected after a centrifugation at 10,000 × *g* at 4 °C for 10 min. Protein concentrations was determined by bicinchoninic acid assay (BCA assay). Fifty micrograms of protein per well were loaded and separated by SDS-PAGE, and were transferred to polyvinylidene difluoride (PVDF) membranes, followed by incubation (i.e. blocking) in bovine serum albumin (BSA) or dry non-fat milk at 25 °C for 1 h. Immunoblot analysis was initiated by incubated with the corresponding antibodies at 4 °C overnight. Cells were then washed three times with 1 × TBST then the blots were incubated with HRP secondary antibody at room temperature for 1 h, and rinsed three times with TBST. Protein bands were detected using enhanced chemiluminescence. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the internal control for protein quantitation.

Luciferase assay

The culture medium was removed from cells, which were then gently rinsed with PBS (Thermo Fisher Scientific) three times. Reporter lysis buffer was added to the plate and cell suspension was harvested with a scraper, centrifuged, and then washed once with PBS. One hundred microliters of reporter lysis buffer per 1 × 10^6 cells were added and the cells was dispersed with a micropipette. The cells were incubated on ice for 20 min followed by centrifugated. The supernatants were collected. One hundred microliters luciferase assay reagent was added and the fluorescence was measured with a luminometer.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Cells were harvested by using TRIzol reagent (Invitrogen, San Diego, CA, USA), total RNA was extracted. The cDNA was constructed using a TaqMan MicroRNA Reverse Transcription kit (4366596; Thermo Fisher Scientific, CN, China), with the miR-96 primer, F-5′-TTTGGCACTAGCACATT-3′. The universal reverse primer was supplied with the miRNA kit. β-Actin was served as an internal control. The miR-96 transcript levels were normalized to β-actin expression and measured using the $2^{-\Delta\Delta Ct}$ method [23].

Statistical analysis

Experiments were performed in triplicate. Data were presented as mean ± standard error of the mean (SEM). Statistical significance was evaluated using GraphPad Prism software (GraphPad Prism, San Diego, CA, USA) using one-way ANOVA followed by Bonferroni test. $P < 0.05$ was considered statistically significant.

RESULTS

Shikonin inhibits cell proliferation and colony formation, downregulates miR-96 expression and upregulates SOCS4 expression in SGC-7901 cells

The effects of shikonin on human GC cell proliferation was determined in SGC-7901 cells using CCK-8 and soft-agar colony formation assays. Shikonin treatment suppressed the proliferation and colony formation of SGC-7901 cells (Figure 1 A and B). Western blot analyses revealed that shikonin significantly reduced the expression levels of PCNA and cyclin D1 in SGC-7901 cells (Figure 1 C). The qRT-PCR assay showed that miR-96 expression was significantly reduced at higher concentrations of shikonin (Figure 1 D). Besides, the protein expression level of SOCS4 was increased by shikonin treatment (Figure 1 E).

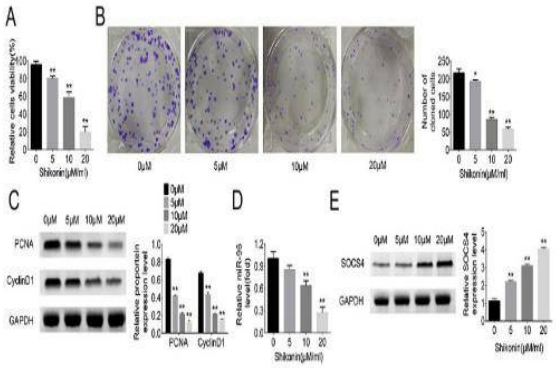

Figure 1: Effect of shikonin on cell proliferation, colony formation, and the expression of miR-96 and SOCS4 expression in SGC-7901 cells. SGC-7901 cells were treated with various concentrations (0, 5, 10 and 20 uM) of shikonin. (A) The viability of SGC-7901 cells. (B) Soft-agar colony formation assay of SGC-7901 cells. (C) Western blot analysis of PCNA and cyclin D1 in SGC-7901 cells. (D) Quantitative qRT-PCR analysis of the relative levels of miR-96 in SGC-7901 cells. (E) The effect of shikonin on expression levels of SOCS4 $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* 0 μM

MiR-96 directly binds to SOCS4 and reduces SOCS4 expression

Further analysis predicted SOCS4 was a potential target of miR-96, and a SOCS4 mutation was generated (Figure 2 A). The luciferase assay showed that the luciferase activity of cells that transfected with miR-96 mimics and SOCS4-wt was significantly lower than that transfected with control mimics and SOCS4-wt (Figure 2 B). The miR-96 expression level was dramatically increased in SGC-7901 cells that transfected with miR-96 mimics (Figure 2 C). Western blot analysis showed that miR-96 mimics led to the inhibition of SOCS4 expression, upregulation of STAT3 phosphorylation, PIM and TAP1 expression (Figure 2 D). These results indicated that miR-96 bound to SOCS4 and regulated the JAK/STAT signaling pathway.

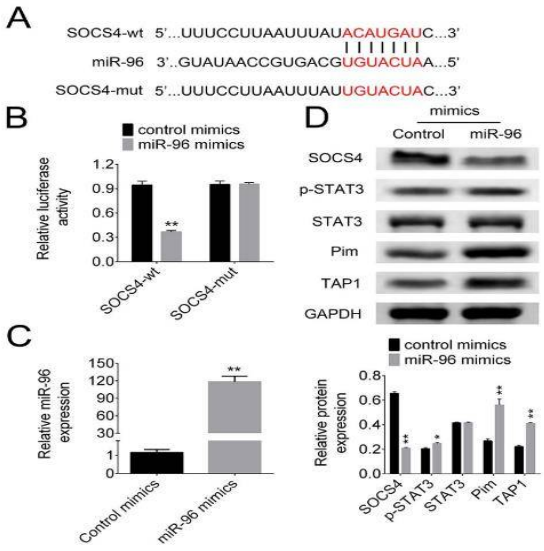

Figure 2: Effects of miR-96 mimics on to the expression of SOCS4, STAT3 phosphorylation, PIM and TAP1. (A) SOCS4 was a potential target of miR-96, predicted by TargetScan. (B) The luciferase activity of cells that co- transfected with miR-96 mimics/ control mimics and SOCS4-wt/ SOCS4-mut; (C) Relative miR-96 expression level in SGC-7901 cells transfected with miR-96 mimics; (D) Expression levels of indicated proteins in SGC-7901 cells transfected with control mimics or miR-96 mimics; $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs* control mimics

Shikonin promotes expression of SOCS4 via inhibiting miR-96 in SGC-7901 cells

For further investigation, the effect of shikonin on the SOCS4 and JAK/STAT pathway-related protein expression levels was determined in SGC-7901 cells transfected with miR-96 mimics. The influence of shikonin on SOCS4 and JAK/STAT pathway-related protein expression levels were reversed by miR-96 mimics (Figure 3), indicating that shikonin modulated the expression levels of SOCS4 and JAK/STAT pathway-related proteins by suppression of miR-96.

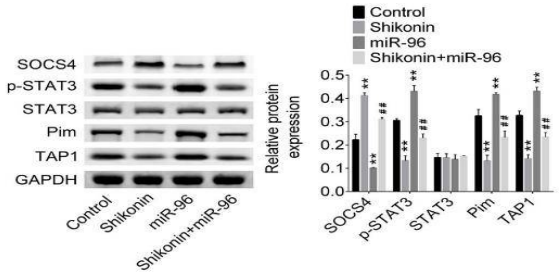

Figure 3: Shikonin promotes the expression of SOCS4 via inhibiting miR-96 in SGC-7901 cells. Western blot analysis of proteins from SGC-7901 cells that transfected with control mimics (control), or treated with 20 μM shikonin (Shikonin), or transfected with miR-96 mimics (miR-96), or transfected with miR-96 mimics with the treatment of 20 μM shikonin (Shikonin+miR-96); $^{**}p < 0.01$ *vs* control, $^{\#\#}p < 0.01$ *vs* miR-96

Shikonin suppresses proliferation and colony formation by mediating miR-96 expression in SGC-7901 cells

Cell proliferation was inhibited by the presence of shikonin, which was reversed by miR-96 overexpression (Figure 4 A and B). The presence of shikonin strikingly downregulated the expression levels of PCNA and cyclin D1, which were completely blocked by miR-96 overexpression (Figure 4 C). These observations suggest that shikonin inhibited cell proliferation and colony formation via modulation of miR-96.

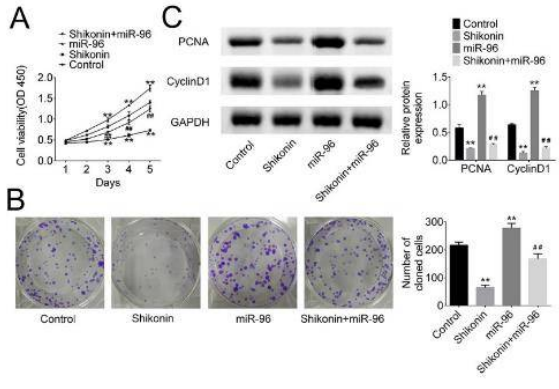

Figure 4: Shikonin suppresses cell proliferation and colony formation via miR-96 in SGC-7901 cells. (A) The proliferation of SGC-7901 cells; (B) Soft-agar colony formation assay of SGC-7901 cells (C) Protein expression levels of PCNA and cyclin D1 in SGC-7901 cells, using western blotting; $^{**}p < 0.01$ *vs* control; $^{\#\#}p < 0.01$ *vs* miR-96

DISCUSSION

Despite the declining incidence of GC, it still ranks as the second highest cancer-related mortality due to poor prognosis [1-3]. Small non-coding, endogenous miRNAs contain about 22 nucleotides (nts) and are important in regulating gene expression at post-transcriptional level via RNA silencing or suppression of translation [12,24]. There is emerging evidence showing that miRNAs play roles in a variety of physiological and pathological processes [12,25]. The miRNAs contribute to human malignancies through a variety of mechanisms such as by mutations involving miRNAs, epigenetic silencing, and dysregulation of transcription [25]. Cellular malignancy depends on dysregulation of miRNA expression. Thus, miRNAs provide opportunities to control oncogenes or tumor suppressor genes, which is a promising strategy for future miRNA-based cancer therapies [25]. Recent studies have reported that herbal medicine exhibits anticancer activities via mediating miRNA expression [12].

Shikonin induces dysregulation of many miRNAs in human endometrioid endometrial cancer cells [12]. The qRT-PCR results revealed that shikonin significantly suppressed miR-96 expression. Consistently, the miR-96 expression was upregulated by chemotherapeutic treatment in SGC7901 cells, which induced chemoresistance, which thus facilitated cell proliferation [26]. In human colorectal cancer, the miR-96 was overexpressed and promoted cell proliferation [27]. MiR-96 was also overexpressed in hepatitis B virus-associated hepatocellular carcinoma, and inhibition of miR-96 blunted the migration and invasion of hepatocellular carcinoma cells [28]. Upregulation of miR-96 may be associated with the aggressive malignancy in bladder cancer [29]. These studies have shown that miR-96 alterations promoted carcinogenesis by mediating the target protein expression in various carcinomas [26-29]. Therefore, miR-96 may act as a biomarker and a therapeutic target for cancer treatment [26-29]. In GC, upregulation of miR-96 was exhibited [30]. However, the regulatory mechanisms of miR-96 in GC are unknown.

In the present study, shikonin inhibited GC cell proliferation via the miR-96/SOCS4 pathway. Shikonin markedly inhibited SGC-7901 cell proliferation. PCNA and cyclin D1 are served as two markers of cell proliferating [31,32], whose expression levels were markedly decreased by shikonin. In consistent with our results, SOCS4 has been identified as a novel cancer suppressor gene which acts as a negative regulator in GC, and its expression level is downregulated in GC [33].

Shikonin treatment dose-dependently upregulated the protein expression level of SOCS4. Luciferase activity assay revealed that miR-96 was directly bound to SOCS4. The overexpression of miR-96 suppressed SOCS4 expression level, increased STAT3 phosphorylation, and promoted the expression of PIM and TAP1, indicating that miR-96 regulated the JAK/STAT signaling pathway, thus promoting carcinogenesis in GC. The overexpression of miR-96 blunted the effects of shikonin treatment on SOCS4 and JAK/STAT pathway-related protein expression levels, suggesting that shikonin induced GC cell apoptosis, at least partially, by inhibiting the JAK/STAT pathway via mediating miR-96/SOCS4 expression. The overexpression of miR-96 also prompted cell proliferation and colony formation, and decreased the expression levels of PCNA and cyclin D1 that were induced by shikonin treatment, showing that shikonin was able to inhibit cell proliferation and colony formation through miR-96 in SGC-7901 cells.

CONCLUSION

The finding is the first evidence demonstrating that shikonin suppressed cell proliferation and colony formation by regulating JAK/STAT pathway via miR-96/SOCS4 pathway in SGC-7901 cells. Thus, miR-96/SOCS4 may provide a potential therapeutic target for GC treatment and prognosis.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. PC designed all the experiments and revised the manuscript. QZ, ZC, FY, RL and TJ performed the experiments.

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