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RETRACTED ARTICLE: Shikonin inhibits proliferation, migration, invasion and promotes apoptosis in NCI-N87 cells via inhibition of PI3K/AKT signal pathway

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ABSTRACT
Shikonin has been testified to wield the anti-tumor action in multiple cancers. But, the clout of Shikonin for gastric cancer (GC) is still inconclusive. The contemplation of this research undertook to disclose the impacts of Shikonin on GC progress meanwhile to uncover the conceivable mechanism. NCI-N87 cells were disposed by Shikonin at diverse concentration points. The trials of CCK-8, colony formation, flow cytometry and Transwell assays were executed for detecting the functions of Shikonin in NCI-N87 cells. The impacts of miR-195 on Shikonin-regulated PI3K/AKT pathway were estimated via western blot. The in vivo trial was detected by xenografts model assay. We found that Shikonin suppressed cell survival and triggered apoptosis in NCI-N87 cells. Additionally, Shikonin restrained cell migration, invasion and down-regulated MMP2, RhoA, ROCK1 and Vimentin expression in NCI-N87 cells. Furthermore, Shikonin notably inhibited PI3K/AKT signal pathway, but the restraining functions were repealed by miR-195 inhibition in NCI-N87 cells. The in vivo trial results revealed that Shikonin determinately impeded tumor formation. In conclusion, these results demonstrated that Shikonin prohibited NCI-N87 cells proliferation, migration, invasion, and accelerated apoptosis by inactivation of PI3K/AKT pathway. The findings maybe provide a fresh opinion for healing GC.

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Introduction
Gastric cancer (GC) is an intermittence tumour-generating from gastric mucosal epithelium [1]. The incidence rate of GC is increasing because of many potential risk factors, such as regional environment, dietary habits, smoking, infections and genetic component [2]. The early symptoms of GC are not obvious, and only a few patients present nausea, vomiting and heartburn [3]. Therefore, it is difficult to pay enough attention to patients. With the development of GC, the patients may appear stomachache, weight loss, difficulty swallowing, hematemesis and other clinical symptoms [4]. The current remedial means of GC mainly comprehend surgery, chemotherapy, radiation therapy and a neoteric approach of biological therapy is being investigated in the clinical trials [5–7]. Recent study has demonstrated that various agents extracted from Traditional Chinese Medicine (TCM) have been proverbially exploited to treat diverse cancers and obtained a good effect [8]. Therefore, drugs from TCM, such as Shikonin, maybe provide a new strategy for healing GC.

Shikonin is an active anthraquinone derivative, which obtains from the root of Lithospermum erythrorhizon [9]. Clinical and pharmacological properties studies have confirmed that Shikonin has anti-inflammation, anti-oxidation, anti-thrombotic and hypoglycemic effects in various inflammatory and infectious diseases [10,11]. It has been showcased that Shikonin gave play to the anti-tumour activity in miscellaneous cancers [12,13]. As Nie et al. revealed that Shikonin retarded liver cancer cell proliferation and expedited apoptosis [14], Zhang et al. divulged that Shikonin could restrain glioblastoma cells migration and invasion through governing PI3K/AKT pathway [8]. However, studies about the effects of Shikonin on GC have not been fully reported.

MicroRNAs (miRNAs) have been testified to be linked to various cancers including GC, which is complicated in adjusting the biological processes of GC cells [15,16]. MiR-195 is a vital miRNA, which has been identified as a predictor of poor prognosis in adrenocortical cancer [17]. Additionally, the attractive researches revealed that miR-195 was linked to the lymph node metastasis and poor prognosis in colorectal cancer [18], as well as could restrain lung cancer progression [19]. Outside of these, the importance of miR-195 in GC also has been reported in the existing researches. The research from Rui et al. disclosed that miR-195 expedited the chemotherapy GC cell sensitivity of cisplatin [20]. Moreover, Wang et al. demonstrated that miR-195 could impede GC cells migration and invasion through modulating basic fibroblast growth factor (bFGF) [21]. Therefore, according to these research
foundations, we attempted to further probe whether miR-195 could regulate the functions of Shikonin in GC cells.

Here on, we first exploited the diverse concentrations of Shikonin to deal with NCI-N87 cells, then the influences in cell growth, migration and invasion were delved. Furthermore, the regulatory functions of Shikonin and miR-195 in PI3K/AKT pathway were further probed to disclose the underlying mechanisms. In the end, the in vivo trial was executed for further exposing the function of Shikonin in GC. These findings maybe emerge a unique evidence for healing GC.

Materials and methods

Cell cultivation and disposition

NCI-N87, a common GC cell line, and GES-1, a human fetal gastric epithelial cell line, were procured from the American Type Culture Collection (ATCC, Manassas, VA, USA). NCI-N87 and GES-1 cells were trained in DMEM (Gibco, Grand Island, NY, USA), which was subsumed 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA, USA) at 37 °C in a drippy environment encompassing 5% CO₂. After culturing, NCI-N87 and GES-1 cells were separated with 0.05% trypsin solution and sub-cultured in 96-well cell culture plates. Shikonin was purchased from Sigma-Aldrich (S7576, St. Louis, MO, USA). Shikonin was melted with DMSO (ATCC) as a reserve solution. Then, the above solution was diluted to configure the concentration to 0, 1, 2, 5, 10 and 20 μM for the related experiments. NCI-N87 cells were dealt with these diverse dosages of Shikonin for 24 h.

Cell viability

NCI-N87 and GES-1 cell suspensions were trained in 96-well plate, and then CCK-8 (Djindo, Gaithersburg, MD) experiment was conducted for measuring cell viability. After administration with dissimilar concentrations of Shikonin, NCI-N87 and GES-1 cells were co-trained with 10 μL of CCK-8 solution in an incubator at persistent 37 °C for 1 h. The optical density (OD) was through conducting a Microplate Reader (Bio-Rad, Hercules, CA, USA) at 450 nm.

Colony formation assay

Five hundred NCI-N87 viable cells were fostered into 6-well plate allowing to culture for 24 h. Subsequently, the above-involved cells were exposed to 10 μM of Shikonin for 24 h. After administration, these disposed cells were then re-suspended in medium and cultivated for 14 days at 37 °C in a 5% CO₂ incubator with 95% air. After this, 0.1% crystal violet (Sigma-Aldrich) was exploited to dye these cells for 10–30 min. The colonies more than 50 cells were counted with applying microscope equipment (Leica Microsystems, Wetzlar, Germany). Each administration was done thrice in this study.

Cell apoptosis assay

To evaluate cell apoptosis, the double staining trial with PI and FITC-conjugated Annexin V (Biosea, Beijing, China) was performed. The NCI-N87 cells were exposed to 10 μM Shikonin for 24 h, which were subsequently laundred twice in PBS solution (Sigma-Aldrich). Next, 5 μL Annexin V-FITC and 10 μL PI were adopted for dying cells for 30 min in the tenebrous condition at indoor temperature. The apoptotic NCI-N87 cells were observed via conducting flow cytometry (Beckman Coulter, Fullerton, CA, USA) equipment.

Migration and invasion assay

NCI-N87 cells were exposed to 10 μM Shikonin for 24 h cultivating, then the capacities of migration and invasion of above-mentioned cells were evaluated via executing 24-well Transwell trial. For cell migration, these administrated cells were trained in the super-culture chamber accompanied by suspending with 200 μL serum-free medium. The substratum chamber was suffused with 600 μL complete medium. These above cells were trained for 24 h at 37 °C and then fastened with 100% methanol (NIST, USA). The migrated cells were dyed for 3 min via exploiting 0.4% crystal violet (Sigma-Aldrich) and counted through utilizing a microscope (Leica Microsystems). The non-migrated cells were cleared through utilizing a wet cotton swab. For cell invasion assessment, the similar methods were preformed, besides the BD Matrigel™ Matrix (BD Biosciences, NY, USA) was employed for coating the inserts.

Cell transfection

NCI-N87 cells were trained in 6-well plates in a 5% CO₂ incubator at 37 °C for 24 h cultivation. Then, the GenePharma Co. (Shanghai, China) synthesized miR-195 inhibitor plasmid and the relevant control (NC) plasmid were transfected into NCI-N87 cells via adopting Lipofectamine 3000 reagent (Invitrogen) in consonance with the reagent specification.

Establishment of xenografts

The special pathogen-free grade BALB/c nude mice (5-week-old) were procured from Vital River (Beijing, China). These selected mice were randomly spilt into three groups, which were managed with Shikonin at 0, 1 and 8 mg/kg. The administrated NCI-N87 cells were gathered and then injected subcutaneously in the right oxter of these laboratory mice. After this, the tumour models were managed with Shikonin via gastric infusion. The diameter of tumours was gauged through utilizing callipers every three days, totally ten times. After four weeks, these laboratory mice were sacrificed, and tumour weight was estimated. This experiment was conducted in conformity with National Institutes of Health (NIH) guidelines and was encouraged by Linyi People’s Hospital.

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

The total RNA procured from NCI-N87 cells, which were managed with Shikonin or transfected with miR-195 inhibitor plasmid was detached via employing Trizol reagent (Invitrogen).
Reverse transcription was conducted to synthesize complementary DNAs (cDNAs) by wielding a Reverse Transcription Kit (Takara, Dalian, China). For miR-195 expression evaluation, the Taqman Universal Master Mix II (ABI, Foster, CA, USA) were capitalized for the determination of miR-195 expression in NCI-N87 cells. U6 was used for normalizing miR-195 expression. These outcomes were figured out through the 2−ΔΔCt method, as described previously (refer figure 1 of ref. [22]).

**Western blot assay**

NCI-N87 cells were administrated with Shikonin at 10 μM for 24 h. After treatment, above cells were laundered three times with PBS to stop the stimulation. Subsequently, these cells were collected and segregated via adopting RIPA lysis buffer (Beyotime, Shanghai, China) with the correlative protease inhibitors (Roche, Basle, Switzerland). The contents of the above-mentioned segregated proteins were appraised through bestowing BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The 20 μg protein samples were segregated through utilizing 10% SDS-PAGE, and next transferred onto PVDF membranes and processed for immunoblotting with the relevant primary antibodies at 4°C all night. The befitting primary antibodies include Cyclin D1 (ab134175, at 1:10,000 dilution), p21 (ab109520, at 1:1000 dilution), Bcl-2 (ab59348, at 1:1000 dilution), Bax (ab32503, at 1:1000 dilution), pro-caspase-3 (ab32499, at 1:10,000 dilution), pro-caspase-9 (ab135544, at 1:100 dilution), cleaved-caspase-3 (ab13847, at 1:1000 dilution), cleaved-caspase-9 (ab2324, at 1:500 dilution), Ras homolog gene family member A (RhoA, ab187027, at 1:5000 dilution), ROCK1 (ab134181, at 1:500 dilution), Vimentin (ab20346, at 1:1000 dilution), phosphorylated (p)-AKT (ab131443, at 1:5000 dilution), AKT (ab32505, at 1:2000 dilution), phosphorylated (p)-PI3K (ab182651, at 1:100 dilution), PI3K (ab40755, at 1:1000 dilution, Abcam, Cambridge, UK), β-actin (ab49900, Abcam, at 1:25000 dilution) was used as an internal control. Then, the membranes were subsequently co-trained with the appropriate second antibodies of goat anti-rabbit IgG (ab205718) and goat-antimouse IgG (ab6789) at a 1:2000 for 1 h at the surrounding temperature. An enhanced chemiluminescent kit (Thermo Fisher Scientific, Inc.) was then used to conduct chemiluminescent detection. The signals were captured and the intensity of the bands was estimated via executing Image Lab™ Software (Bio-Rad).

**Statistical analysis**

The consequences in this study were described as the mean ± SD. The Graphpad Prism 5.0 statistical software (San Diego, CA, USA) was employed for the evaluation of the statistical analysis data. Comparisons between multiple groups were computed via capitalizing ANOVA with Tukey post-hoc test. The p < .05 was considered that this consequence had significant.

**Results**

**Shikonin frustrated cell proliferation in NCI-N87 cells**

For the exploration of the activity of Shikonin in GC cells proliferation, we foremost appraised the effect of Shikonin on GES-1 cells. We discovered that Shikonin crippled the viability of GES-1 cells (p < .05) at the dosages of 10 and 20 μM (Figure 1(A)). No obvious impact of Shikonin on GES-1 cells viability at the dosages of 1, 2 and 5 μM. These data indicated that Shikonin at the dosages of 1, 2 and 5 μM had no toxicity to cells. Then, NCI-N87 cells were handled with the dissimilar dosages of Shikonin (0, 1, 2, 5, 10 and 20 μM) and cell viability was evaluated through executing CCK-8 experiment, again. As shown in Figure 1(B), Shikonin significantly inhibited cell viability at the dosages of 5 μM (p < .05), 10 and 20 μM (p < .01). No obvious function of Shikonin in cell viability at the dosages of 1 and 2 μM. Owing to the inhibitory action of Shikonin at 10 μM in cell viability was higher than that at 5 μM; therefore, we selected 10 μM of Shikonin for the subsequent experiments.

Next, colony formation experiment was performed. In Figure 1(C), the colonies were observably diminished by Shikonin administration at 10 μM (p < .05). Furthermore, Cyclin D1 and p21 expression were detected via western blot trial. We observed that Shikonin reduced Cyclin D1 level, while increased p21 level in NCI-N87 cells (p < .001, Figure 1(D)). Above observations revealed that Shikonin prohibited cell proliferation in NCI-N87 cells.

**Shikonin promoted cell apoptosis in NCI-N87 cells**

For the investigation of the function of Shikonin in cell apoptosis, flow cytometry assay was conducted. As displayed in Figure 2(A), cell apoptosis of NCI-N87 cells was prominently elicited by (p < .01). In addition, western blot outcomes revealed that Shikonin notably obstructed Bcl-2 expression, quickened Bax and cleaved-caspase-3/-9 expression. The pro-caspase-3 and pro-caspase-9 expression have no changed by Shikonin stimulation (Figure 2(B)). These data indicated that Shikonin hastened cell apoptosis in NCI-N87 cells.

**Shikonin obstructed cell migration and invasion in NCI-N87 cells**

Next, Shikonin functioned in the migratory ability of NCI-N87 cells was determined via applying Transwell experiment. In Figure 3(A), cell migration of NCI-N87 cells was memorably restricted by Shikonin at 10 μM (p < .01). Besides, Shikonin (10 μM) clearly curtailed MMP2, RhoA, and ROCK1 in NCI-N87 cells (p < .001, Figure 3(B)). Likewise, Shikonin functioned in the invasive ability of NCI-N87 cells was also appraised through employing Transwell experiment. We discovered that cell invasion was also abated by Shikonin (10 μM, p < .01, Figure 3(C)). Further, Vimentin protein level was memorably declined by Shikonin (10 μM, p < .05, Figure 3(D)). The above-mentioned observations illustrated that Shikonin restrained cell migration and invasion in NCI-N87 cells.
Shikonin discouraged PI3K/AKT pathway in NCI-N87 cells

Above results indicated that Shikonin could restrain NCI-N87 cells proliferation, migration, invasion, meanwhile provoke apoptosis. So, we supposed that Shikonin affected these cellular biological processes maybe through regulation of relevant signal pathway. To confirm this hypothesis, western blot trial was executed for the evaluation of the roles of Shikonin in PI3K/AKT pathway. In Figure 4(A,B), the phosphorylated AKT and PI3K protein levels were visibly retarded by Shikonin in NCI-N87 cells (p < .001). There was no regulatory function of Shikonin in PI3K and AKT protein levels. The results indicated that Shikonin thwarted PI3K/AKT pathway in NCI-N87 cells.

Shikonin thwarted PI3K/AKT pathway via modulating miR-195

The pertinence between Shikonin and miR-195 was evaluated via working out qRT-PCR experiment. As depicted in Figure 5(A), miR-195 expression was clearly ascended by Shikonin in NCI-N87 cells (p < .01). For further exploration of the impacts of miR-195 on Shikonin-inactivated PI3K/AKT pathway, miR-195 inhibitor and its negative control were transfected into NCI-N87 cells. Results disclosed that miR-195 expression was distinctly diminished in miR-195 inhibitor-transfected NCI-N87 cells (p < .01, Figure 5(B)). Western blot result revealed that miR-195 inhibition significantly repealed the repressive impact of Shikonin on PI3K/AKT pathway (Figure 5(C,D)). All these above-correlated discoveries attested that Shikonin rescinded PI3K/AKT pathway via governing miR-195 expression in NCI-N87 cells.

Shikonin suppressed tumour formation in vivo

For further confirmation of the anti-tumour function of Shikonin in GC, we examined the tumour diameter and weight in vivo via executing Xenograft tumour model experiment. In Figure 6(A), the tumour diameter was significantly decreased by Shikonin (p < .05 or p < .01). Similarly, Shikonin remarkably
declined the tumour weight as contrasted with control group ($p < .05$ or $p < .01$, Figure 6(B)). In sum, the relevant discoveries revealed that Shikonin restrained tumour formation in vivo, further hinting the anti-tumour activity of Shikonin in GC.

**Discussion**

Based on the results from the current study, we disclosed that Shikonin overtly inhibited cell proliferation, migration, invasion, and elicited apoptosis in NCI-N87 cells. Western blot assay revealed the regulation effect of Shikonin on cell cycle, apoptosis and metastasis-correlated factors. Moreover, we observed that Shikonin notably inhibited PI3K/AKT signal pathway, but the suppressive impacts were crippled by miR-195 suppression in NCI-N87 cells. Furthermore, in vivo experiment results indicated that Shikonin significantly suppressed tumour formation.

Shikonin (also known as Zicao) is a common herbal medicine, which has been used to treat miscellaneous cancers, encompassing lung cancer [23], pancreatic cancer [24] and endometrial cancer [25]. Recent study demonstrated that Shikonin exerted anti-tumour activity by suppression of cell proliferation. For example, one study reported that Shikonin could suppress skin carcinogenesis cell proliferation [26]. Another study demonstrated that Shikonin inhibited cell proliferation and altered cell cycle in colon cancer cells [27]. Additionally, Kim et al. reported that Shikonin could suppress cell proliferation via arresting cell cycle at G2/M phase by regulation of p21 expression [28]. Despite the repressive activity of Shikonin in cell proliferation in diverse tumour cells have been proverbially reported, the action of Shikonin in cell GC cells proliferation remains hazy. We discovered that Shikonin inhibited cell proliferation by suppressing cell viability, decreasing colony formation and modulating Cyclin D1 and p21 protein levels. Our study may provide a neoteric evidence for the anti-proliferative activity of Shikonin in GC.

Cell apoptosis is an important manifestation of cell death, which is implicated with the development of tumours [29].
Bcl-2 family and caspase family are key regulators in this process [30]. In view of recent study, the anti-tumor effect of Shikonin is closely related to cell apoptosis [31]. As Tian et al. demonstrated that Shikonin evidently induced cell apoptosis by decreasing Bcl-2, increasing Bax and enhancing the activities of caspase-3 and caspase-9 expressions in squamous cell carcinoma (SCC) cells [32]. In terms of GC, Hyeonseok et al. corroborated that Shikonin quickened cell apoptosis by adjusting p53 and Nrf2 in stomach carcinoma cells [33]. Lee et al. found that Shikonin triggered GC cells apoptosis via generation of reactive oxygen species [34]. Similar with above-mentioned researches, we found that Shikonin remarkably evoked cell apoptosis by hindering Bcl-2 and ascending Bax, cleaved-caspase-3 and cleaved-caspase-9 expression in NCI-N87 cells.

Figure 5. Effect of miR-195 on Shikonin-regulated PI3K/AKT signal pathway in NCI-N87 cells. The dosage of Shikonin at 10 μM was employed to dispose NCI-N87 cells. (A) qRT-PCR experiment results revealed that Shikonin ascended miR-195 expression in NCI-N87 cells; Then, NCI-N87 cells were transfected with miR-195 inhibitor and NC. (B) qRT-PCR experiment results displayed that miR-195 inhibition decreased miR-195 expression in NCI-N87 cells; (C) Western blot assay revealed that Shikonin blocked PI3K/AKT pathway via enhancement of miR-195 (D). **p < .01; ***p < .001; ns: no significance.

Figure 6. Shikonin suppressed tumor formation in vivo. The different concentrations of Shikonin (0, 4 and 8 mg/kg) were used to treat NCI-N87 cells. (A) Xenograft tumor model assay results displayed that Shikonin suppressed the tumor diameter; (B) Xenograft tumor model assay results revealed that Shikonin declined the tumor weight. *p < .05; **p < .01.
At present, tumour metastasis has become a vital clinical obstacle and a great challenge in the healing of various cancers [35]. It is well known that migration and invasion are involved in adjusting cancer cell malignant and metastasis [36]. Emerging evidence proved that Shikonin could impede glioma cells migration and invasion via repression of MMP-2 and MMP-9 [8]. Similarly, Jiang et al. found that Shikonin prohibited breast cancer cells migration and invasion through inhibition of MMP-9 [37]. As described above, our study also demonstrated that Shikonin could restrain NCI-N87 cells cell migration and invasion via hindering MMP2, RhoA, ROCK1 and Vimentin expression. The result similar with Liu et al. corroborated that Shikonin frustrated cell invasion and migration in GC cells [38].

PI3K/AKT pathway presents a conspicuous role in mediating cell proliferation, apoptosis, migration and invasion [39]. Mounting evidence indicated that Shikonin triggered cell apoptosis via adjusting PI3K/AKT pathway in cervical cancer and endometrial cancer cells [40,41]. However, whether Shikonin exerts anti-tumour action via modulation of PI3K/AKT pathway has not been investigated. We revealed that Shikonin hampered PI3K/AKT pathway in NCI-N87 cells. Recent study revealed that miR-195 joined in mediating tumour growth of GC cells [42]. Our study found that miR-195 suppression rescinded the impeding function of Shikonin in PI3K/AKT pathway.

More importantly, to further prove the anti-tumour impacts of Shikonin on GC, we contrasted the Xenograft tumour model to appraise tumour diameter and tumour weight. The results displayed that Shikonin remarkably suppressed tumour diameter and weight, indicating that Shikonin restrained tumour formation in vivo. These data further clarified the anti-tumour activity of Shikonin in GC. However, further studies are still necessary to delve the cross-regulatory functions of Shikonin and miR-195 in GC in vitro and in vivo.

Altogether, these findings indicated that Shikonin frustrated NCI-N87 cells proliferation, migration, invasion and expedited apoptosis. Moreover, Shikonin inactivated PI3K/AKT signal pathway by adjusting miR-195. These findings maybe supply a newfangled insight for GC treatment.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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