NWK inhibits growth and induces apoptosis in MC cells through suppression of epithelial-mesenchymal transition via the AKT/GSK-3β/β-catenin pathway

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Abstract
AKT/GSK-3β/β-catenin signaling pathway plays an important role in the progression of Precancerous lesions of gastric cancer (PLGG). Niweikang has achieved good results in the treatment of chronic atrophic gastritis with intestinal metaplasia, atypical hyperplasia and other PLGC, and the treatment rate is more than 83%. However, traditional Chinese medicine lacks deep mechanism in the treatment of chronic atrophic gastritis. The present study investigated the effect of NWK-containing serum on MC cells and the AKT/GSK-3β/β-catenin signaling. Proliferation assay, migration and invasion and flow cytometry demonstrated Niweikang-containing serum dose-dependently inhibited cell viability. Western blot analysis found that NWK-containing serum effectively modulated molecules related to apoptosis and AKT/GSK-3β/β-catenin signaling. In conclusion, our findings indicate that NWK-containing serum inhibits growth and induces apoptosis in MC cells through suppression of AKT/GSK-3β/β-catenin pathway, suggesting that NWK-containing serum may have potential for use in PLGC treatment.

Keywords: Precancerous lesion of gastric cancer, Traditional Chinese medicine, Serum-containing Niweikang, AKT/GSK-3β/β-catenin signaling pathway

Summary:
What is already known about this subject:
2. Niweikang-containing serum can inhibit the proliferation, migration and invasion of MC cells.
3. Niweikang-containing serum inhibits the invasive and migratory abilities of MC cells through the inhibition of EMT by targeting the Akt/GSK-3β/β-catenin pathway.
4. Niweikang is a promising Chinese medicine compound, which is very effective in the treatment of PLGC.

What are the new findings:
2. Niweikang-containing serum can more accurately evaluate the pharmacological effects of Chinese herbal compound in vivo.
3. Our results highlighted Niweikang potential as an adjuvant therapy for the treatment of PLGC.
Introduction

Epithelial-mesenchymal transformation (EMT) refers to the biological process of epithelial cells transforming into mesenchymal phenotype cells, which plays an important role in embryonic development, tissue regeneration, wound healing, organ fibrosis and tumor progression.[1] Studies have shown that EMT is closely related to the occurrence and development of gastric cancer, and widely exists in the process of precancerous lesions of gastric cancer (PLGC).[2] EMT is associated with a variety of signaling pathways and gene expression disorders, which are mainly manifested in cell morphological changes and abnormal expression of related proteins.[3] The epithelial cells of gastric mucosa changed from irregular polygon to fusiform, the adhesion between cells decreased and the arrangement was disordered. The expression of E-cadherin in labelled epithelial cells decreased, while the expression of N-cadherin, Vimentin and α-SMA in labelled interstitial cells increased.

Traditional Chinese medicine (TCM) has unique advantages in the treatment of chronic diseases, and in regulating immunity, reducing toxicity, enhancing efficiency, preventing relapse, inhibiting metastasis and improving the quality of life.[4] Therefore, the strategy of using traditional Chinese medicine to treat PLGC is urgent clinical needs.[5] However, some drugs only exert their pharmacological effects through metabolism, and crude extracts of Chinese medicine can not accurately evaluate the changes of drugs in vivo.[6] Serum pharmacology is a scientific analysis method for extracting serum from oral Chinese medicine.[7] This method can avoid the tedious procedures of the purification of the monomers to some extent. Moreover, it can exclude the interference which is unique and difficult to determine in the study of the activity of the crude extract, making the result more reliable.[8]

During EMT, the decrease of E-cadherin level may lead to the accumulation of β-catenin, which leads to the transfer of β-catenin from the cytoplasm to the nucleus, and the formation of beta-catenin/LEF complex with transcription factor LEF in the nucleus, thus activating the Wnt/β-catenin signaling pathway.[9, 10] β-catenin is a key molecule in the Wnt/β-catenin signaling pathway, which promotes the transcription of multiple carcinogenic target genes related to cancer progression.[11] β-catenin is also a bifunctional protein involved in EMT, which improves cell adhesion by attaching E-cadherin to the cytoskeleton. In addition, β-catenin acts as a transcription factor to activate downstream target genes, such as C-myc.

Wnt/β-catenin signaling pathway is involved in the development of a variety of tumors. β-catenin is the most critical channel transfer molecule in the Wnt signaling pathway, and the transfer of β-catenin to the cell nucleus is a sign of the activation of the pathway.[12, 13] Notably, activated AKT leading to decreased phosphorylation of AKT substrates, phosphorylated Akt is known to induce an inactive form of GSK-3β by phosphorylating its Ser9 residues, and β-catenin is also modulated by GSK-3β, thereby activating Wnt/β-catenin signaling pathway.[14-16] Although GSK-3β is involved in the PI3K/Akt and Wnt/β-catenin signaling pathways, this study has little research in PLGC. [17] The drug targeting the signaling pathway is expected to become a new idea for the development of oncology drugs.

Niweikang (NWK)’s compound was summed up by clinical experience for many years in our hospital. It has achieved good effect in the treatment of precancerous lesions such as chronic atrophic gastritis with intestinal metaplasia and atypical hyperplasia, as well as adjuvant treatment of gastric cancer after operation. The effective rate is over 83%. Previous studies in the project group found that NWK can improve the inflammation of gastric mucosa in CAG model rats, promote the regeneration of glands, increase microcirculation blood flow, and promote the repair of local gastric mucosa.

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To further explore the mechanism of Niweikang in preventing and treating PLGC, MC cells (MNNG-induced malignant transformation of human gastric epithelial cell line (GES-1) were used as the model. There have been many breakthroughs regarding the upstream regulatory mechanisms of AKT; however, whether AKT/GSK-3β/β-catenin signaling is regulated by NWK-containing serum in PLGC has not been explored. In this study, the aim was to investigate the effects of NWK on the proliferation, invasion and migration of MC cells and the underlying mechanisms involved.

Materials and methods

Animals. A total of 60 healthy male Wistar rats (age, 7-8 weeks), weighing 250±20 g, were purchased from Beijing China Fukang Biological Technology Co., Ltd. (no. SCXK 2009 0007; Beijing, China).

Cell lines and cell culture. Normal gastric mucosal cell line GES-1 was purchased from the cell resource center of the GES-1 cells is presented by Professor Qi Xin, Department of molecular biology, Ocean University of China. Cells were cultured in Roswell Park Memorial Institute (1640) supplemented with 10% fetal bovine serum (FBS) at 37°C with a 5% CO2 atmosphere in a humidified incubator (Thermo Fisher Scientific, Waltham, MA, USA).

Reagents and instruments. Fetal bovine serum (FBS), RIP cell lysate, PVDF membrane and ECL chemiluminescence reagents were purchased from Millipore company, RPMI1640 medium and double resistance (penicillin and streptomycin sulphate) were purchased from Hyclone company, CCK-8 kit and apoptosis detection kit were bought from Japan Dolindo company, and Rabbit anti human PCNA, Bcl-2, Bax, and Caspase-3, AKT, P-AKT Ser473, β-catenin, GSK-3β and P-GSK-3β Ser9 were purchased from Cell Signaling company; Rabbit anti human GAPDH purchased from Elabscience company; horseradish peroxidase labelled Sheep anti rabbit two was bought from Cell Signaling company.

Experimental drugs. Hemsleya amabilis Diels(Xue Dan), Astragalus mongholicus Bunge (Huang Qi), Lobaria retigera (Bory) Trevis (Lao Longpi), Polygonum Cillinerve (Nakai) Ohwi (Zhu Shaqi), Scutellaria barbata D. Don (Ban Zhilian), Cleistocactus sepium (Kunth) A.Weber (Hai Piaoxiao), Citrus reticulate Blanco (Chen Pi), Zingiber officinale Roscoe (Sheng Jiang) were purchased from Hangzhou Hu Qingyu Tang Pharmaceutical Co., Ltd.

Preparation of Niweikang. Hemsleya amabilis Diels(15 g), Astragalus mongholicus Bunge (10 g), Lobaria retigera (Bory) Trevis (10 g), Polygonum Cillinerve (Nakai) Ohwi (3 g), Scutellaria barbata D. Don (15 g), eistocactus sepium (Kunth) A.Weber (10 g), Citrus reticulate Blanco (6 g), Zingiber officinale Roscoe (6 g) were prepared as follows(water extraction method):2 times were extracted and 1 h was extracted and the ratio of solid to liquid was 12 times, followed by concentration of the supernatant under ordinary pressure, centrifugation at 2,000 x g for 15 min, and sterilization by filtration. The final standardized product consisted of 2 g crude medicine per milliliter. Wei Fu Chun powder (formula: rabdosia, Fructus aurantii, ginseng) is provided by Hangzhou Hu Qingyu Tang Pharmaceutical Co., Ltd.

Preparation of drug-containing serum. After three days of acclimation, 60 Wistar rats were divided at random into three groups, including the low-dose of NWK (L, n=10), middle-dose of NWK (M, n=10), high-dose of NWK (H, n=10), WFC (P, n=10) and blank control (BC, n=10) groups. Rats received an intragastric middle-dose of NWK (3.94g/kg, which corresponds to 10 times the adult clinical dose, and low-dose of NWK=middle-dose of NWK/2, high-dose of NWK=middle-dose of NWK*2), WFC (1.23 mg/kg, which corresponds to 10 times the adult clinical dose), or an equal volume of Normal saline, twice a day for ten days. Abdominal aortic blood (5-10 mL) was collected following the final drug administration. Blood samples were allowed to stand at 4°C for 4 h, followed by centrifugation at 3000 x g for 10 min at 4°C. Sera were carefully extracted by suction and then incubated in a 56°C water bath for 30 min to inactivate complements and antibodies present in the sera. Subsequent to 0.22 μm filter-sterilization, the sera were stored in sterile centrifuge tubes at -20°C.

All experiments were conducted at the Department of Medical Research center and the medical animal laboratory of the Affiliated Hospital of Qingdao University, Qingdao, Shan- dong, P. R. China. This study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University and conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Establishment of MC cell model

GES-1 cells(2×105/well) in logarithmic growth phase were inoculated in a 6 cm dish, cultured overnight, treated with 2×105 mol/L MNNG for 24 hours, and then replaced with normal complete medium for 48 hours. The morphological changes of the cells after injury were observed under inverted microscope. The model cells are called MC cells.

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Cell viability assay

The cells (5×103/well) were seeded in 96-well plates and incubated for 24 h. When the cell density reached 60-70%, MC cells were treated with 10% BC, L, M, H and P group for 24, 48, and 72 h. Cell viability was assayed via CCK-8 assay.

Colony formation assay

MC cells were treated with 10% NC, L, M, H, P group for 24 h. The cells were then cultured in 6-well plates (3×103 cells/well), and the medium was changed every 2 d for 12-14 days. The colony-forming efficiency of single cells was calculated as the number of colonies/number of inoculated cells×100 and is reported as a percentage.

Cell apoptosis analyses

The Alexa Fluor 488 annexin V/dead cell apoptosis kit was used to identify apoptotic 10% BC, L, M, H, P group-treated MC cells. The data were analyzed using FlowJo software (version7.6).

Transwell migration and invasion assays

Cell migration and invasion were assessed using Transwell polycarbonate membranes (8.0 μm pores; Corning Inc., Corning, NY, USA) placed in each well of a 24-well plate containing 600 μl 1640 with 10% FBS. For migration, MC cells were treated with 10% BC, L, M, H, P group for 24 h and then seeded (2.0×105 cells) on the membranes using serum-free 1640 for 24 h at 37°C. To assess invasion, the Transwell membranes were precoated with Matrigel (BD, Franklin Lakes, NJ, USA). After the 24-h incubation, the cells were fixed with methanol for 15 min and stained with 0.5% crystal violet for 15 min. The percentage of cells that had penetrated through the membrane was quantified under a microscope at × 200 magnification.

Western blotting.

Total proteins were extracted from cells using RIPA lysis buffer and the concentrations were determined with a BCA protein quantitation kit. Equal amounts of protein from samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto 0.45 μm polyvinylidene difluoride membranes. Subsequently, the membranes were blocked with 5% milk in PBS plus 0.1% Tween 20 (TBST) for 60 min. The membranes were incubated with primary antibodies (at the recommended dilutions) overnight at 4°C. The next day, the membranes were incubated for 60 min with the corresponding secondary antibodies. The bands were detected using an enhanced chemiluminescence reagent and visualized with a Fusion FX7 System. ImageJ software was used to calculate the intensity (gray value) of each protein band, which was normalized to that for GAPDH.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 7.0 software. All experiments were performed in triplicates. Data were analyzed by one-way analyses of variance (ANOVAs) and are presented as means±SDs. A P value of <0.05 was considered as statistically significant.

Results

MNNG induced morphological changes and EMT of GES-1 cells

The GES-1 cells of normal gastric mucosa injured by MNNG were observed by inverted microscope. The results showed that the cells in blank group had regular morphology and few floating cells; in the model group, there were more dead cells floating in the supernatant of the cell culture medium, and the adherent cells showed obvious morphological changes. The cells lost their original epithelial-like morphology, became irregular long spindle-shaped, and some giant cells appeared (Figure 1A). This indicated that MNNG could obviously damage GES-1 cells, and the induced cells are called MC cells.

In order to verify whether EMT occurs in induced cells, the related proteins of induced cells were analyzed. The results showed that the cells in blank group had regular morphology and fewer floating cells; in the model group, there were more dead cells floating in the supernatant of the cell culture medium, and the adherent cells showed obvious morphological changes. The cells lost their original epithelial-like morphology, became irregular long spindle-shaped, and some giant cells appeared (Figure 1A). This indicated that MNNG could obviously damage GES-1 cells, and the induced cells are called MC cells.

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Figure 1. MNNG induces EMT changes in GES-1 cells. (A) Morphology changes in GES-1 cells after exposure to MNNG. (B) MNNG induces GES-1 cells EMT change. Western blotting was performed to determine the EMT biomarker protein expression levels of E-cadherin, β-catenin, vimentin, and N-cadherin. Data are expressed as the means ± SDs from three separate experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. GES-1 (magnification, x20).

Containing serum inhibits the proliferation of MC cells

To study the effect of different groups containing serum on the viability of MC cell was treated for 24, 48, and 72 h. Different groups containing serum inhibited the proliferation of MC cells in a dose- and time-dependent manner (Figure 2A). We next investigated the anticancer effect of containing serum on MC cells. The cloning formation assay showed that the number of cell colonies in the different groups containing serum-treated MC cell group was lower than that of the BC group (Figure 2B). Compared with BC group, the number of cells in each group was: BC group, 73.00±10.23 (P<0.001), 39.67±4.92 (P<0.01), 21.33±2.87 (P<0.001), 34.00±5.10 (P<0.001). We found that containing serum was the most effective at inhibiting cell colony formation at a concentration of H group. We also measured the expression of PCNA by Western blotting and found that protein levels were decreased in the different groups containing serum-treated MC cells, and the difference was statistically significant (Figure 2C). Compared with BC group, the relative expression of PCNA protein in L, M, H and P groups decreased after 48 hours of treatment with Niiweikang drug-containing serum, which were: 0.46±0.029 (P<0.01), 0.12±0.022 (P<0.001), 0.057±0.024 (P<0.001), 0.20±0.022 (P<0.001).

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Different groups containing serum inhibited the proliferation of MC cells, treated for 24, 48, and 72 h in a dose- and time-dependent manner. B. The colony formation ability was decreased after treatment with different groups containing serum. C. Representative Western blot and quantitation of PCNA expression showing a dose-dependent decrease with different groups containing serum treatment. Data are expressed as the means ± SDs from three separate experiments. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 vs. BC.

**Containing serum promotes apoptosis of MC cells**

Different groups-containing serum significantly induced cell apoptosis after 48 h of treatment as determined by flow cytometry (Figure 3A). Different doses of serum containing Niweikang could obviously promote the apoptosis of MC cells. With the increase of dose, the apoptotic rate increased significantly. Compared with BC group, there was significant difference in L, M, and P group by 15.70±1.51 (P<0.05), 22.88±1.77 (P<0.01), 22.50±0.94 (P<0.01), and apoptotic cells in H group increased significantly by 27.19±0.81 (P<0.001). We also investigated the expression of apoptosis-related proteins by Western blotting and observed increases in Bax, and caspase-3 and decreased in Bcl-2. Compared with BC group, the expression of Bcl-2 protein in each group was 0.41 ±0.033 (P>0.05), 0.27 ±0.043 (P<0.05), 0.19 ±0.051 (P<0.01), 0.46 ±0.029 (P>0.05), but L group and P group had no significant statistical significance (P>0.05). While the expression of Bax protein was 0.33±0.046 (P>0.05), 0.36±0.0047 (P<0.05), 0.46±0.049 (P<0.01), 0.44±0.031 (P<0.01), but L group and P group had no significant statistical significance (P>0.05); the expression of Caspase protein was 0.54±0.066 (P > 0.05), 0.68±0.067 (P<0.05), 0.78±0.033 (P<0.01), 0.71±0.045 (P <0.01), and there was no significant difference in L group (P>0.05). The results suggest that containing serum promotes both early and late stage apoptosis in containing serum -treated MC cells.

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Figure 3. Different groups containing serum induces the apoptosis of MC cells. A. The effect of different groups containing serum on cell apoptosis of MC cells was analyzed using Annexin V/PI and the apoptosis percentage represented in histogram. B. Western blot of bcl-2, bax, and caspase-3 expression showing a dose-dependent decrease with different groups containing serum treatment. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 vs. BC.

**Containing serum inhibits the migration and invasion of MC cells**

After treating MC cells with different concentrations of different groups containing serum for 48 h, cell migration was lower than that of the blank controls (Figure 3A) Compared with BC group, the number of MC cells in L, M, H and P groups decreased by 145±7.87 (P<0.05), 63.72±4.99 (P<0.01), 26.67±4.50 (P<0.0001) and 65.65±6.18 (P<0.01), and the migration of MC cells was inhibited by the drug-containing serum of NWK in a concentration-dependent manner. Moreover, cell invasion (Figure 3B) was decreased in different groups containing serum-treated MC cells compared with that in BC group, the number of MC cells in L, M, H and P groups decreased by 67±7.79 (P<0.05), 47±6.38 (P<0.01), 23.67±3.09 (P<0.001) and 40.33±6.55 (P<0.01). These decreases corresponded with reduced expression of the migration- and invasion-related proteins MMP-2 and MMP-9 (Figure 3C). Thus, different groups containing serum dose-dependently inhibits GC invasion and migration. Compared with BC group, the expression of MMP-9 protein in each group was 0.67±0.078 (P<0.05), 0.45±0.049 (P<0.01), 0.39±0.021 (P<0.001) and 0.42±0.033 (P<0.01).

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There was a significant difference among the groups treated with the drug-containing serum of NWK. The expression of MMP-2 protein was 0.68±0.031 (P>0.05), 0.47±0.045 (P<0.05), 0.40±0.040 (P<0.01), 0.57±0.021 (P<0.05), respectively. There was a significant difference among the groups treated with the drug-containing serum of NWK.

**Effects of NWK-containing serum on the protein expression related to AKT/GSK-3β/β-catenin signaling pathway**

At the junction of normal epithelial cells, E-cadherin and beta-catenin form complex to maintain the stability of epithelial cytoskeleton and promote cell-to-cell interaction.[18] The down-regulation or deletion of E-cadherin expression is considered to be the key link in inducing EMT, and along with the increase of N-cadherin expression, the induction of beta-catenin into nucleus is involved in gene transcription and regulation.[9, 19] According to the above results, the migration and invasiveness of MC cells were inhibited after treatment with the NWK-containing serum. Therefore, we speculate that the NWK-containing serum inhibits the EMT of MC cells. Western blot analysis showed that NWK-containing serum could down-regulate the expression of N-cadherin, vimentin and α-SMA in MC cells in a dose-dependent manner, and up-regulate the expression of E-cadherin in MC cells (Fig.5B). These results suggest that NWK can inhibit the EMT process of MC cells. Compared with BC group, the expression levels of E-cadherin protein increased by 1.22±0.074 (P>0.05), 1.35±0.090 (P>0.05), 1.55±0.073 (P<0.01), 1.17±0.10 (P<0.05); on the contrary, the expression of N-cadherin, vimentin and alpha-SMA decreased. The expression levels of N-cadherin protein decreased to 2.28±0.17 (P>0.05), 2.25±0.14 (P<0.05), 2.17±0.15 (P<0.05) and 2.63±0.063 (P>0.05), and vimentin protein decreased to 1.13±0.15 (P>0.05), 1.04±0.055 (P>0.05), 0.89±0.080 (P<0.05), 1.69±0.20 (P>0.05), respectively. They were 0.25±0.012 (P>0.05), 0.26±0.014 (P>0.05), 0.25±0.036 (P<0.01), 0.18±0.0154 (P<0.05).

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It has been confirmed that carcinogenic serine/threonine kinase AKT regulates EMT in many tumors. AKT inhibits the transcription of E-cadherin gene, resulting in the decrease of E-cadherin protein expression, thus loosening the intercellular junction. In order to verify whether AKT can be regulated by Niweikang, we conducted a Western blotting. The results showed that Niweikang decreased the expression of phosphorylated AKT (p-AKT), especially in the high dose group (Fig. 5A). Compared with BC group, the expression levels of p-AKT Ser473 protein in each group were 0.26±0.025 (P<0.05), 0.26±0.0047 (P<0.05), 0.14±0.012 (P<0.001), 0.22±0.029 (P<0.01). The results showed that Niweikang could significantly inhibit the expression of p-AKT Ser473 protein in a concentration-dependent manner.

Studies have shown that phosphorylated AKT inhibits GSK-3β activity by phosphorylating its Ser9 residue, accompanied by accumulation of β-catenin in the nucleus and activation of Wnt pathway. In this study, we found that the phosphorylation level of GSK-3β in MC cells was down-regulated in high-dose group compared with BC group. Therefore, the expression level of p-GSK-3β Ser9 protein were 0.35±0.029 (P>0.05), 0.38±0.050 (P>0.05), 0.21±0.0045 (P<0.05), 0.37±0.0045 (P>0.05). The results showed that Niweikang could inhibit the expression of p-GSK-3β Ser9 protein only in high dose group. However, compared with BC group, the expression of GSK-3β and AKT protein in each group had no significant difference (P>0.05).

The expression of E-cadherin was inhibited, and the complex of β-catenin/E-cadherin on cell membrane was destroyed, resulting in a large number of β-catenin entering the nucleus. Therefore, we studied the effect of NWK-containing serum on the expression of β-catenin protein. Compared with BC group, the expression level of β-catenin protein were 0.31±0.051 (P>0.05), 0.42±0.033 (P<0.05), 0.24±0.033 (P<0.05), 0.22±0.033 (P<0.05). The results showed that L group had little effect on the expression of β-catenin protein in MC cells, but the serum concentration of Niweikang in M, H group and significantly inhibited the expression of β-catenin protein (Fig. 5A).

Figure 5. Different groups containing serum suppresses the EMT process by targeting the Akt/GSK-3β/β-catenin pathway. (A) Western blot analysis of protein related to apoptosis and AKT/GSK-3β/β-catenin signaling. (B) Western blot assays were used to assess the effects of NWK-containing serum on EMT process.

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To further clarify the relationship between NWK-containing serum inhibiting MC cell proliferation and AKT/GSK-3β/β-catenin pathway, GSK690693, H, and GSK690693+H was used to treat MC cells for 24 hours. The results showed that GSK690693 up-regulated the phosphorylation level of AKT at the Ser473 site in MC cell, but the expression of AKT had no statistical significance (P>0.05). The phosphorylation level of GSK-3β and the expression level of β-catenin protein in MC cells were decreased by GSK690693 treatment (Fig.6A). However, the expression of GSK-3β and AKT protein in each group had no significant difference. It is suggested that Niweikang may inhibit Akt/GSK-3β/β-catenin pathway by inhibiting phosphorylation of AKT and GSK-3β.

The ser473 site p-AKT level of MC cells decreased and the ser9 level of p-GSK-3β Ser9 further decreased in the H and GSK690693 groups. In addition, the expression of β-catenin protein was also inhibited by 0.79±0.057 (P<0.001), 0.55±0.054 (P<0.001), 0.32±0.037 (P<0.0001).

GSK690693 also inhibited the occurrence of EMT in MC cells (Fig.6B). Western blotting results also showed that the use of H and GSK690693 group could more effectively increase E-cadherin and decrease N-cadherin, vimentin and α-SMA in MC cell. Overall, these results suggest that the serum containing Niweikang inhibits the EMT process of MC cells by regulating the Akt/GSK-3β/β-catenin pathway.

Figure 6. The relationship between Niweikang-containing serum and the Akt/GSK-3β/β-catenin pathway using the Akt inhibitor. (A) Western blot analysis of protein related to apoptosis and AKT/GSK-3β/β-catenin signaling. (B) Western blot assays were used to assess the effects of NWK-containing serum on EMT process.

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Discussion

In clinic, traditional Chinese medicine has a certain anti-cancer effect on tumor, and has advantages of low side effects, low cost, cheap, improving the quality of life and so on, so it is easy to be accepted by patients.\[20, 21\] Traditional Chinese medicine has been widely used to treat various diseases in medical practice. Here, we demonstrate the antitumor effects of NWK-containing serum on apoptosis, proliferation, migration, and invasion of MC cells in vitro, consistent with results from previous studies.

The main mechanism of Bax to promote cell apoptosis is to induce cytochrome C to release from mitochondria to cytoplasm, and to activate the downstream Caspase protein family, thus promoting the occurrence of cell apoptosis.\[22, 23\] Bcl-2 is mainly located in the mitochondrial membrane.\[24\] By maintaining the stability of the mitochondrial membrane and preventing the release of cytochrome C, it plays a role in inhibiting apoptosis and can form two polymers with Bax to antagonize the apoptosis effect of Bax protein. It can be seen that the initiation of apoptosis is regulated by Bcl-2 family proteins.\[25, 26\] Caspase is the most common pathway of apoptotic signal transduction, and many pathways that regulate apoptosis are ultimately anti-apoptotic or apoptosis induced by the function of Caspase enzymes.\[22\] Caspase-3 is the key regulator of the apoptosis response of tumor cells.\[27\] After mitochondria damage, the release of cytochrome C and activation of Caspase-3 to activate cells apoptosis can be promoted by Bax, and Caspase-3 can be activated by promoting the release of cytochrome C.\[28, 29\] The results suggest that the expression of Bcl-2 in MC cells can be reduced by lowering the expression of apoptosis factor related to mitochondrial pathway, and the expression of Bax and Caspase-3 can be increased and the mitochondrial pathway is activated. The downstream Caspase-3 protein suggests that NWK-containing serum may inhibit apoptosis by regulating the related factors of mitochondrial pathway. Nevertheless, further research is needed to identify whether apoptosis-induction in MC cell by NWK-containing serum depends on the extrinsic (death receptor), or the intrinsic (mitochondrial) pathway, or both.

And we found the NWK-containing serum treatment also increased the percentages of early and late apoptotic MC cells. The present study using flow cytometry showed that NWK-containing serum treatment of H group for 48 h remarkable increased apoptosis rates of MC cells. The results indicated that induction of cell apoptosis might contribute to the growth inhibition effects on MC cells by NWK-containing serum.

The ability to prevent metastasis is an important effect of anticancer therapeutics.\[4\] The migration and invasion of cancer cells are influenced by the extracellular matrix.\[4\] MMPs is the main speed limiting enzyme that regulates the metabolism of intercellular matrix (ECM).\[30\] It can degrade ECM specifically and participate in a variety of physiological and pathophysiological processes, and it is a large family. MMP-2 and MMP-9 are the related factors of tumor migration, which are involved in tumor migration and invasion, and they can degrade gelatin, laminin and type IV collagen, which play an important role in the invasion and metastasis of many malignant.\[31-33\] The study found that the expression of MMP-2 and MMP-9 in gastric cancer increased significantly.\[34\] The results of western blot analysis showed that NWK-containing serum significantly decreased the expression of MMP-2 and MMP-9. These results suggest that NWK-containing serum can inhibit the migration and invasion of MC cells, and its mechanism may be related to the inhibition of MMP-2 and MMP-9 expression.

This study found that the expression of E-cadherin in epithelial cells decreased after MNNG treatment, while the expression of N-cadherin and Vimentin in mesenchymal cells increased, suggesting that radiotherapy induced EMT in MC cells. More importantly, the expression of E-cadherin increased and the expression of N-cadherin, vimentin and α-SMA decreased after treatment with Niweikang. Therefore, these results suggest that Niweikang may enhance the sensitivity of cancer cells to MC cell by inhibiting EMT formation of radiotherapy cells.

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The occurrence of gastric cancer is involved in a variety of signal pathways and interlaced with each other to form a network configuration. The maladjustment of Akt/GSK-3β/β-catenin is one of the main factors for the occurrence of gastric cancer, and the target of Akt/GSK-3β/β-catenin signal for the treatment of gastric cancer has also become a hot spot in recent years.[35, 36] The treatment of various drug pathways and target molecules can effectively inhibit the proliferation, invasion and metastasis of gastric cancer cells and the tumor in vivo and in vivo. GSK-3β is one of the main targets in the downstream Akt, and it has a variety of physiological functions.[3, 37] Moreover, it can phosphorylate a series of substrates, including promoting apoptosis promoting factors, inhibiting the survival of the transcription factors and activating the apoptosis related protein kinase, which is the main component of various signal pathways in the cells.[3, 38] Akt can reduce GSK-3β through the phosphorylated Ser9 site, and then influence the expression and activity of β-catenin.[39] β-catenin is the key factor in the Wnt signaling pathway, which can effectively transfer the extracellular signal to the nucleus.[13, 16] The accumulation of the expression level can affect the expression of the target gene in the nucleus, thus regulate the transcription of DNA and the process of protein synthesis and participate in the process of apoptosis. The expression of β-catenin is closely related to the activity of GSK-3β, and is negatively regulated by upstream GSK-3β.[40] Therefore, the results in the present study suggested that the molecular mechanism of inhibiting proliferation and inducing apoptosis by NWK may lie on suppression of the AKT/GSK-3β/β-catenin pathway. Our results showed that NWK-containing serum markedly decreased the phosphorylation of Ser9 residues of GSK-3β respectively. This indicated that NWK-containing serum relieved the inhibition of GSK-3β by downregulating the phosphorylation of Akt, and may have a regulatory role in the expression and location of β-catenin. Western blotting was performed to examine the effect of NWK-containing serum on the expression and location of the β-catenin protein. We found that both H group of NWK-containing serum significantly inhibited β-catenin expression. These results confirm that Akt/GSK-3β/β-catenin is an objective pathway by which NWK-containing serum in MC cells.

In conclusion, our findings indicate that NWK inhibits growth and induces apoptosis in MC cells through suppression of epithelial-mesenchymal transition via the AKT/GSK-3β/β-catenin pathway, suggesting that NWK may possess potential possibility for use in PLGC treatment.

References

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