Wulong Xiaozheng Wan medicated serum inhibits epithelial-mesenchymal transition in human gastric carcinoma cell line BGC823 by modulation of transforming growth factor-β1/Smad signaling

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OBJECTIVE: To evaluate the effect of Wulong Xiaozheng Wan medicated serum on the epithelial-mesenchymal transition (EMT) of BGC823 cell induced by transforming growth factor-β1 (TGF-β1) and to explore its mechanism.

METHODS: EMT model of BGC823 was stimulated by TGF-β1. Wulong Xiaozheng Wan medicated serum and LY-364947 were used as intervention. The proliferation and adhesion of BGC823 were detected by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide and flow cytometry was used to detect the apoptosis. The invasion and migration were detected by Transwell. The level of matrix metalloproteinases was detected by enzyme-linked immunosorbent assay. The expressions of related proteins and mRNA of EMT marker and TGF-β1/Smad signal pathway were detected by Western blot and reverse transcription-polymerase chain reaction.

RESULTS: Compared with the TGF-β1 group, Wulong Xiaozheng Wan medicated serum could inhibit the ability of proliferation, heterogeneous adhesion, invasion, and migration. It also promotes apoptosis and homotypic adhesion in BGC823, with a dose-dependent manner. Meanwhile, Wulong Xiaozheng Wan medicated serum could regulate the expression of related proteins and mRNA of TGF-β1/Smad signaling pathway, and inhibit the expressions of EMT transcription factors and EMT markers.

CONCLUSION: Wulong Xiaozheng Wan medicated serum inhibited epithelial-mesenchymal transition by down-regulated the expression of TβRI and the activation of TGF-β1/Smad signaling pathway.

Keywords: Transforming growth factor beta1; Smad proteins; Signal transduction; Epithelial-mesenchymal transition; Matrix metalloproteinases, secreted; BGC823 cell; Wulong Xiaozheng Wan

INTRODUCTION
Gastric cancer, one of the common tumors of the diges-
Epithelial-mesenchymal transition (EMT) refers to the process of transformation of epithelial cells into mesenchymal cells with characteristics of high migration, invasion, anti-apoptosis and degradation of extracellular matrix, which is the first and key step of invasion and metastasis of cancer cells, and is closely related to the occurrence, progression and drug resistance of gastric cancer.\(^7\) EMT is mainly regulated by a number of transcription factors, including Slug, Snail, and Twist, and accompanied by the down-regulation of epithelial cell markers such as E-cadherin and the up-regulation of mesenchymal cell markers such as N-cadherin and Vimentin.\(^5\) Studies have confirmed that the ability of proliferation, invasion and migration enhanced significantly with the occurrence of EMT in gastric cancer cells.\(^3\) Therefore, effective suppression of EMT has become the focus of research in the prevention and treatment of gastric cancer.

Transforming growth factor-β (TGF-β) has been proved to be one of the most widely bioactive polypeptide cytokines mediating EMT. TGF-β phosphorylates downstream Smad 2 and Smad 3 proteins by recognizing and binding the receptor TGF-β on the surface of cell membrane, and promotes recognition of MH2 domain of Smad 4 protein to form heterodimer complexes, which then transport to the nucleus and bind to sequence-specific DNA binding proteins, activate specific target genes, promote differentiation and apoptosis, inhibit cell growth, and regulation of EMT by mediating G1 phase arrest.\(^13\) Smad 7, an antagonist of TGF-β/Smad signaling pathway, inhibits the activation of TGF-β/Smad signaling pathway by regulating the activity of TβRI kinase and down-regulate the phosphorylation of Smad 2 and Smad 3.\(^15\) Therefore, the development of natural anti-cancer substances has become one of the important strategies in the field of cancer research. Wulong Xiaozheng Wán (WLXZW) is a new anti-cancer drug consisted of ethanolic extracts of Qinglongyi (Exocarpium Juglandis Immunatum) and Ciwujia (Radix et Caulis Acanthopanacis Santicosi). Qinglongyi (Exocarpium Juglandis Immunatum) is the immature pericarp of Juglans regia and Jugland mandshurica Maxim, and widely distributed in the northeast of Asia. It has been used as a Chinese herbal medicine for thousands of years and has significant cytotoxic activity against cancer cells.\(^17\) Ciwujia (Radix et Caulis Acanthopanacis Santicosi) is a precious traditional Chinese herbal medicine and mainly distributed in the northern part of China. The main active ingredients of Ciwujia (Radix et Caulis Acanthopanacis Santicosi) are Acanthoside, Acanthoside E, Acanthopanax Senticosus Saponin, and Acanthopanax Senticosus Polyaccharides, which have been proved to have the effects of anti-fatigue, anti-aging, and anti-cancer.\(^19\) Therefore, human gastric cancer BGC823 cell induced by TGF-β, was used to further elucidate the anti-tumor effect and mechanism of WLXZW in this study. The changes of proliferation, apoptosis, adhesion, invasion, and migration of BGC823 cell were observed. The expression of EMT markers and related proteins and genes in the TGF-β/Smad signaling pathway were detected by Western-blot and reverse transcription-polymerase chain reaction (RT-PCR).

**MATERIALS AND METHODS**

**Materials**

WLXZW was provided by the manufacturing laboratory of Heilongjiang Academy of Traditional Chinese Medicine (Batch number: 20160811, Harbin, China); RPMI1640 medium, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), LY-364972 (TGF-β Type I Receptor Kinase Inhibitor) were purchased from Sigma-Aldrich LLC. (St. Louis, MO, USA); TGF-β1 was purchased from R&D Systems Inc. (Minneapolis, MN, USA); 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was purchased from AMRESCO LLC. (Fountain Parkway Solon, OH, USA); Boyden Chamber Transwell was purchased from Corning Inc. (Corning, NY, USA); Matrigel was purchased from Becton, Dickinson, and Company. (Franklin Lakes, NJ, USA). Primary antibody: mouse anti-human monoclonal antibody of TβRI, Smad 2, Smad 7, Twist, matrix metalloprotein (MMP)-2, MMP-7, MMP-9, N-cadherin, Vimentin, E-cadherin, Snail, and β-actin were obtained from R&D Systems, Inc. (Minneapolis, MN, USA), rabbit anti-human monoclonal antibody of Smad 3, phospho-Smad 2\(^\text{517}^{\text{a}}\), phospho-Smad 3\(^\text{517}^{\text{a}}\), and Slug were purchased from Abcam Inc. (Cambridge, MA, USA). Secondary antibodies: horseradish peroxidase labeled goat anti-rabbit and anti-mouse IgG were purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, PA, USA). AnnexinV-FITC/PI double staining, MMP-2, MMP-7, and MMP-9 kits were purchased from Nan-
jing Jiancheng Institute of Bioengineering (Nanjing, China). Standard substances of Syringoside and Chlorogenic acid were purchased from Shanghai Jiaoguang Biotechnology Co., Ltd. (Batch No. 111574-201604 and 110753-201615, Shanghai, China), and standard substances of Acanthoside E and Isofraxidin were purchased from Sigma-Aldrich LLC. (Batch numbers: BCBQ7879V and BCBN9268V, St. Louis, MO, USA). The standard substance of Juglone was obtained from Chengdu Reifenis Biotechnology Co., Ltd. (Batch number: H-075-131230, Chengdu, China). Methanol and acetonitrile were purchased from TEDIA Inc. (Phoenix, AZ, USA).

**Determination of the activity of WLXZW**

WLXZW was smashed and sifted, then precision weighed 1.0 g to 25 mL volumetric bottle. Standard substance solution was obtained by ultrasound treatment (power 500 W, frequency 40 kHz) for 30 min after adding an appropriate amount of methanol, constant volume, through the microporous membrane. Meanwhile, the appropriate amount of Syringoside, Chlorogenic acid, Acanthoside E, Isofraxidin, and Juglone were precisely weighed to 25 mL volumetric bottle, the mixed standard substance solution of Syringoside 1.375 mg/mL, Chlorogenic acid 1.634 mg/mL, Acanthoside E 0.835 mg/mL, Isopyridine 0.463 mg/mL, and Juglone 0.674 mg/mL were prepared by ultrasound treatment, constant volume, and microporous membrane, with methanol as control sample solution. Chromatographic conditions: Lichrospher 5HE C18 (4.6 mm × 250 mm × 5 μm), mobile phase: acetonitrile (A)-0.5% phosphoric acid solution (B) gradient elution, flow rate of 0.5 mL/min, detection wavelength: 265 nm, column temperature: 30 °C, injection volume of 10 mL. The mixed standard substance solution, the sample solution, and the control solution were determined according to the chromatographic conditions.

**Preparation of medicated serum**

Twenty-four rats were randomly divided into control group, WLXZW low, medium, and high dose group (drug concentrations were 0.74, 0.82 and 0.91 mg/kg), WLXZW groups were treated with corresponding dosage of drug suspension, and the control group was given equal volume of distilled water. Rats were given gavage therapy once a day for 7 d, during which the rats were fed with normal diet. One hour after the last gastric perfusion, abdominal aortic blood was collected. After 1 h of standing at room temperature, 3600 r/m was centrifuged for 10 min. The supernatant was absorbed and inactivated at 56 °C for 30 min, then stored at −80 °C for use.

**Cell lines and groups**

Human gastric cancer cell BGC823 was purchased from Cell Resource Center, IBMS, CAMS/PUMC. Cells were cultured in RPMI1640 medium containing 10% calf serum in a constant temperature incubator with 5% CO₂ at 37 °C, then digested by trypsin, and passed and harvested. The number of living cells was calculated by trypansome blue staining, and the cells in logarithmic growth phase were used for research. Cells were divided into five groups: control group (blank serum), TGF-β1 group (TGF-β1, 10 ng/mL + blank serum), low, medium and high dose group of WLXZW (TGF-β1, 10 ng/mL + medicated serum of WLXZW concentration of 0.74, 0.82 and 0.91 mg/mL respectively), and LY364947 group (positive drug control, TGF-β1, 10 ng/mL + blank serum + LY3649472 μM).

**Proliferation of BGC823 cell by MTT**

Cells in logarithmic growth phase at a concentration of 6 × 10⁴/mL were inoculated in 96 well culture-plates with 6 compound holes in each group. After cell adherence growth, the culture medium was removed and the corresponding serum culture medium was replaced. The cells were cultured 24, 48, 72, and 96 h at 37 °C and 5% CO₂. Before the end of the experiment, 20 μL MTT solution was added to each pore, and then the culture was continued for 4 h. Absorbed the culture medium and DMSO 150 μL was added. After the crystals were fully dissolved, the absorbance value (OD) at 490 nm was determined by an enzyme labeling instrument, and the inhibition rate of cell proliferation was calculated. Inhibition rate = (1 - OD Blank group/OD Control group) × 100%.

**Apoptosis of BGC823 cell by flow cytometry**

Cells in logarithmic growth phase at a concentration of 1 × 10⁴/mL were inoculated in 96 well culture-plates with 6 compound holes in each group. 24 h after the various treatments, the BGC823 were collected into EP tube and centrifuged at 4 °C and 3000 r/min for 10 min. After being rinsed with phosphate buffered saline (PBS) twice, the cells were resuspended in 500 μL of binding solution. After filtration, the cells were transferred into another tube for antibody labeling: 8 μL of PI and 4 μL of AnnexinV-FITC were added at room temperature in darkness and the specimens were detected using a flow cytometer in 5 min.

**Homogeneous adhesion of BGC823 cell by MTT**

BGC823 cell in logarithmic growth phase were inoculated in 24 well culture-plate. At the time of cell fusion, 7 × 10⁴ BGC823 cell treated with corresponding serum for 24 h were inoculated into 96 well culture-plate. Cells were incubated in incubators at 37°C and 5% CO₂ for 2 h, then the culture medium was sucked out, and PBS was used to wash out the non-adherent cells. 20 μL MTT solution was added to each pore, and then the culture medium was continued for 4 h. After that, the culture medium was abandoned, 150 μL DMSO was added, and the OD value was measured at 490 nm with a micro-oscillator oscillating for 10 min.
**Heterogeneous adhesion of BGC823 cell by MTT**

Under sterile condition, 24-well plate coated with matrigel diluted with RPMI 1640 medium. Dried at 37 °C overnight, then washed the board. Dried for 1 h at room temperature, 100 µL 2% BSA was added to incubate for 1 h, then washed the board. 7 × 10^5 BGC823 cells treated with corresponding serum for 24 h were inoculated into 96 well culture-plates, and then adhesion in the incubator for 1 h. The non-adherent cells were carefully washed out with PBS and then replaced with a serum-free medium of 100 µL. At the same time, 10 µL 0.5% MTT was added for 4 h. After that, the culture medium was absorbed, 150 µL DMSO was added and dissolved by shock for 10 min. The OD value at 490 nm was determined by an enzyme labeling instrument.

**Invasion of BGC823 cell by transwell**

Matrigel was evenly coated on the microporous membrane in transwell chamber at 4 °C and placed overnight at 37 °C for curing. After 5 min of placement of the complete culture medium, the culture medium was sucked and discarded, then placed in 6-well culture plate. 100 µL BGC823 treated with corresponding serum for 24 h were inoculated in the upper transwell chamber at a concentration of 5 × 10^{6} cells/mL, and 600 µL medium containing 10% FBS was added into the lower chamber, then incubated in 37 °C, 5% CO₂ incubators for 24 h. Removed transwell, sucked out upper chamber liquid, fixed with methanol at room temperature for 40 min, wiped off superfluous cells on upper chamber surface with a cotton swab, inverted and air-dried. 0.1% crystal violet dyeing solution was added and then washed with the tap water slowly after 30 min, inverted and air-dried, and observe under the microscope. The average and standard deviation of the number of perforating cells in 10 different visual fields were counted under each 200-fold microscope. The relative number of invasive cells is used to express the invasive ability of tumor cells.

**Migration of BGC823 cell by transwell**

Totally 100 µL BGC823 cell treated with corresponding serum for 24 h were inoculated in the upper transwell chamber at a concentration of 5 × 10^{6} cells/mL, and 600 µL medium containing 10% FBS was added into the lower chamber, then incubated in 37 °C, 5% CO₂ incubator for 24 h. Removed transwell, sucked out upper chamber liquid, fixed with methanol at room temperature for 40 min, wiped off superfluous cells on upper chamber surface with a cotton swab, inverted and air-dried. 0.1% crystal violet dyeing solution was added and then washed with the tap water slowly after 30 min, inverted and air-dried, and observe under the microscope. The average and standard deviation of the number of transmembrane cells in 10 different visual fields were counted under each 200-fold microscope. The relative number of migrating cells is used to express the migration ability of tumor cell.

**MMP-2, MMP-7, and MMP-9 in BGC823 cell by Enzyme-linked immunosorbent assay**

Cells in logarithmic growth phase were cultured in 6-well plate with 100 µL at a concentration of 1 × 10^{7}/mL. After 24 h of culture, the serum-free medium was replaced by starvation for 24 h. Each pore was cultured with 100 µL corresponding drug-containing serum and blank serum. After 72 h of culture, the supernatant was collected and centrifuged and transferred to the centrifugal tube for reserve. The OD value was determined by enzyme labeling at 440 nm after coloring. The concentration of MMP-2, MMP-7, and MMP-9 was calculated.

**Detection of Western blot**

BGC823 cell treated with corresponding serum for 24 h were washed with PBS twice, and 50 µL RIPA cell lystate was added. The cells were fully lysed on ice for 30 min and centrifuged at 12 000 r/min at 4 °C for 15 min. The supernatant was collected and the protein concentration was determined by the bulked segregant analysis (BSA) method. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 30 mg protein samples. After transmembrane, polyvinylidene difluoride membrane was sealed with 5% skimmed milk powder for 1 h and incubated overnight at 4 °C with primary antibody. Tris buffered saline tween (TBST) was used to wash the membrane three times, incubated at room temperature for 2 h after adding the corresponding second antibody, and washed 3 times with TBST. The electrochemiluminescence (ECL) chemiluminescent liquid was used for color detection, and β-actin was used as internal reference. The data were processed by Quantity One, and the relative expression level of protein was expressed by measuring the gray ratio of protein band to internal reference protein band.

**Detection of RT-PCR**

BGC823 cell were treated with corresponding serum for 24 h, then digested and collected. Total RNA was extracted by Trizol method. The concentration of RNA was determined by nucleic acid-protein quantitative analyzer. The integrity of RNA was detected by agarose gel electrophoresis, and cDNA was transcribed by reference to the reverse transcription kit. Primer sequences are presented in Table 1. The PCR products were photographed by 2% agarose gel electrophoresis and photographed by gel imager. The gray values of β-actin, Smad 2, Smad 3, Smad 7, N-cadherin, Vimentin, E-cadherin, Vimentin, N-cadherin, Vimentin, E-cadherin, and β-actin were measured by Image J software.

**Statistical analysis**

Statistical analysis was was carried out with SPSS 20.0 software (IBM, Armonk, NY, USA). The measurement
data of each group were expressed as mean ± standard deviation. One-way analysis of variance was used for comparison among groups, and the least significant difference test was used in pairwise comparison among multiple groups. The difference was statistically significant with \( P < 0.05 \).

**RESULTS**

**Determination of the activity of WLXZW**
The result showed that the numbers of theoretical plates of Syringoside, Chlorogenic acid, Acanthoside E, Isofraxidin, and Juglone were no less than 4000 (Figure 1).

**Proliferation of BGC823 cell**
Compared with the control group, the OD value of proliferation at 24, 48, 72, and 96 h increased significantly after stimulation with TGF-\( \beta \) (\( P < 0.01 \)). The OD value of proliferation decreased significantly (\( P < 0.05 \) or \( P < 0.01 \)) and the inhibition rate of proliferation increased significantly (\( P < 0.01 \)) after the treatment of WLXZW medicated serum at each dosage and LY364947 at each time point when compared with the TGF-\( \beta \) group (Figure 2). The inhibiting effect of WLXZW medicated serum was time-dependent and dose-dependent, especially in the high-dose group of WLXZW medicated serum at 96 h.

**Apoptosis of BGC823 cell**
Apoptosis was significantly inhibited after TGF-\( \beta \) stimulation (\( P < 0.01 \)), while WLXZW medicated serums in three dose group could significantly increase the apoptosis of BGC823 cells with a dose-dependent manner (\( P < 0.01 \)). LY364947 showed the same trend as WLXZW medicated serum (Figure 3).

**Adhesion of BGC823 cell**
The homogeneous adhesion decreased significantly while the heterogeneous adhesion increased significantly after stimulation with TGF-\( \beta \) when compared with the control group (\( P < 0.01 \)). The homogeneous adhesion of BGC823 cells decreased significantly and the heterogeneous adhesion increased significantly (\( P < 0.01 \)) after the treatment of WLXZW medicated serums and LY364947, and the effect of high-dose WLXZW medicated serum on BGC823 cells was the most obvious (Figure 4).

**Invasion and migration of BGC823 cell**
When compared with the control group, the numbers of BGC823 cell invading and migrating to the sub-

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![Image](https://example.com/image1.png) **Figure 1 High Performance Liquid Chromatography of WLXZW**

Figure 2 Effect of WLXZW on proliferation of BGC823 cell
A: comparison of OD value of proliferation at different time points treated with WLXZW medicated serum; B: comparison of inhibition rate of proliferation at different time points treated with WLXZW medicated serum. Control group (blank serum), TGF-β group (TGF-β: 10 ng/mL + blank serum), low, medium and high dose group of WLXZW (TGF-β: 10 ng/mL + medicated serum of WLXZW concentration of 0.74, 0.82 and 0.91 mg/mL respectively), LY364947 group (TGF-β: 10 ng/mL + blank serum + LY364947 2 μM). OD: optical density; WLXZW: Wulong Xiaozheng Wan; TGF-β: transforming growth factor-β. Compared with control group, \( P < 0.01 \); compared with TGF-β group, \( P < 0.01 \).

Figure 3 Effect of WLXZW on the apoptosis of BGC823 cell
A: effect of WLXZW medicated serum on the apoptosis of BGC823 cells at 24 h; B: flow cytometry showed the apoptosis of BGC823 cell in each group. B1: control group (blank serum); B2: TGF-β group (TGF-β: 10 ng/mL + blank serum); B3: TGF-β + WLXZW low does group (TGF-β: 10 ng/mL + medicated serum of WLXZW concentration of 0.74 mg/mL); B4: TGF-β + WLXZW medium does group (TGF-β: 10 ng/mL + medicated serum of WLXZW concentration of 0.82 mg/mL); B5: TGF-β + WLXZW high does group (TGF-β: 10 ng/mL + medicated serum of WLXZW concentration of 0.91 mg/mL); B6: LY364947 group (TGF-β: 10 ng/mL + blank serum + LY364947 2 μM). WLXZW: Wulong Xiaozheng Wan. Compared with control group, \( P < 0.01 \); compared with TGF-β group, \( P < 0.01 \).
membrane increased significantly after stimulation of TGF-β1 ($P < 0.01$). The numbers of invasion and migration decreased significantly after the intervention of WLXZW medicated serums and LY364947 ($P < 0.01$), and showed a dose-dependent effect (Figure 5).

**Expressions of MMP-2, MMP-7, and MMP-9**

The degradation of the extracellular matrix is the basis of migration of tumor cells. As the main component of extracellular matrix degradation, MMPs play a key role in the invasion and migration of tumors. As shown in Figure 6A-C, when compared with the control group, the levels of MMP-2, MMP-7, and MMP-9 in BGC823 cells increased significantly after stimulation of TGF-β1 ($P < 0.01$). The levels of MMP-2, MMP-7, and MMP-9 induced by TGF-β1 could be significantly reversed in each dose group of WLXZW medicated serum and LY364947, the difference was statistically significant when compared with the TGF-β1 group ($P < 0.05$ or $P < 0.01$). The high-dose group of WLXZW medicated serum showed the best effect. At the same time, the results of Western blot were consistent with those of Elisa (Figure 6D).

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**Figure 4** Effect of WLXZW on adhesion of BGC823 cell
A: effect of WLXZW medicated serum on homogeneous adhesion of BGC823 cell at 24 h; B: effect of WLXZW medicated serum on heterogeneous adhesion of BGC823 cell at 24 h. Control group (blank serum), TGF-β group (TGF-β: 10 ng/mL + blank serum), low, medium and high dose group of WLXZW (TGF-β: 10 ng/mL + medicated serum of WLXZW concentration of 0.74, 0.82 and 0.91 mg/mL respectively), LY364947 group (TGF-β: 10 ng/mL + blank serum + LY364947 2 μM), WLXZW: Wulong Xiaozheng Wan; TGF-β: transforming growth factor β. Compared with control group, $P < 0.01$; compared with TGF-β group, $P < 0.01$.

**Figure 5** Effect of WLXZW on invasion and migration of BGC823 cell
A: effect of WLXZW medicated serum on invasion and migration of BGC823 cell at 24 h were detected by transwelling. B1, B7: control group (blank serum); B2, B8: TGF-β group (TGF-β: 10 ng/mL + blank serum); B3, B9: TGF-β1 + WLXZW low does group (TGF-β: 10 ng/mL + medicated serum of WLXZW concentration of 0.74 mg/mL); B4, B10: TGF-β1 + WLXZW medium does group (TGF-β: 10 ng/mL + medicated serum of WLXZW concentration of 0.82 mg/mL); B5, B11: TGF-β1 + WLXZW high does group (TGF-β: 10 ng/mL + medicated serum of WLXZW concentration of 0.91 mg/mL); B6, B12: LY364947 group (TGF-β: 10 ng/mL + blank serum + LY364947 2 μM). WLXZW: Wulong Xiaozheng Wan; TGF-β: transforming growth factor β. Compared with control group, $P < 0.01$; compared with TGF-β group, $P < 0.01$. 

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**Figure 6**
A: effect of WLXZW medicated serum on invasion and migration of BGC823 cell at 24 h; B: Invasion and migration of BGC823 cell.
**TGF-β/Smad signaling pathway**

The expressions of TβRI, p-Smad 2, and p-Smad 3 in BGC823 cell increased significantly, and the expression of Smad 7 decreased significantly (P < 0.01), while the expressions of Smad 2 and Smad 3 were not significantly changed when compared with the control group after stimulation of TGF-β. The expressions of TβRI, p-Smad 2, and p-Smad 3 in BGC823 cell decreased significantly and the expression of Smad 7 increased in each dose group of WLXZW medicated serum and LY364947, the differences were statistically significant when compared with the TGF-β1 group (P < 0.05 or P < 0.01). The high dose group of WLXZW medicated serum showed the best therapeutic effect (Figure 7).

**EMT-related proteins**

As an important inducer of EMT, the results of western blot confirmed that TGF-β could significantly down-regulate the expression of E-cadherin protein and up-regulate the expressions of N-cadherin, Vimentin, Slug, Snail, and Twist protein in BGC823 cells when compared with the control group (P < 0.01). WLXZW medicated sera in three dose group and

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**Figure 6 Effect of WLXZW on MMP-2, MMP-7, and MMP-9 of BGC823 cell**

A-C: MMP-2, MMP-7 and MMP-9 in BGC823 cell by Elisa at 24 h; D: MMP-2, MMP-7 and MMP-9 in BGC823 cell by Western blot at 24 h; E: relative expression level of protein was expressed by measuring the gray ratio of protein band to internal reference protein band. 1: control group (blank serum); 2: TGF-β group (TGF-β, 10 ng/mL + blank serum); 3: TGF-β1+WUXZ low does group (TGFB-1, 10 ng/mL + medicated serum of WLXZW concentration of 0.74 mg/mL); 4: TGF-β1+WUXZW medium does group (TGF-β, 10 ng/mL + medicated serum of WLXZW concentration of 0.82 mg/mL); 5: TGF-β1+WUXZ high does group (TGF-β, 10 ng/mL + medicated serum of WLXZW concentration of 0.91 mg/mL); 6: LY364947 group (TGF-β, 10 ng/mL + blank serum + LY364947 2 μM). WLXZW: Wulong Xiaozheng Wan; TGF-β: transforming growth factor-β; MMP: matrix metalloprotein. Compared with control group, *P < 0.01; compared with TGF-β group, **P < 0.05; P < 0.01.
LY364947 could significantly up-regulate the expression of E-cadherin protein and down-regulate the expressions of N-cadherin, Vimentin, Slug, Snail, and Twist protein in BGC823 cells when compared with the TGF-β group (P < 0.05 or P < 0.01). With the increase of the concentration of WLXZW, the effect of regulation on EMT-related proteins increased gradually (Figure 8).

**TGF-β/smad signaling pathway and EMT-related mRNA**

As shown in Figure 9A, the results of RT-PCR were consistent with those of Western blot. The expressions of TβRI, Smad 2 and Smad 3 mRNA in BGC823 cells were up-regulated and the expression of Smad 7 mRNA was down-regulated after TGF-β stimulation (P < 0.01). WLXZW medicated serum in all dose groups could significantly up-regulate the expression of Smad 7 mRNA when compared with the TGF-β group (P < 0.01). WLXZW medicated serums in three dose group could significantly up-regulate the expression of E-cadherin mRNA and down-regulate the expressions of N-cadherin, Vimentin, Slug, Snail, and Twist mRNA in BGC823 cells when compared with the TGF-β group (P < 0.05 or P < 0.01). In addition, LY364947 also showed the same action trend as the WLXZW medicated serums (Figure 9B-H).

**DISCUSSION**

EMT is a rapid and reversible process of cell transformation, which is determined by mutations of certain alleles in the cell genome and interstitial signals (especially interstitial signals at the junction of the cell and the matrix), these allele mutations and interstitial signals change the gene expression of intracellular signaling pathway and further induce the transformation of cell phenotype by acting on specific receptors on cell surface. In the process of phenotypic transformation, epithelial cells lose polarity and acquire fibroblast-like properties. At the meanwhile, the adhesion structure of
epithelial cells was destroyed, which was manifested by the reorganization of cytoskeleton composed of actin microfilaments in cells and the disappearance of anchoring junction, tight junction, cytokeratin, and desmosome-intermediate filament, and the destruction of these structures further led to the significantly enhanced proliferation, invasion, and migration.23,24

The tumor microenvironment is the internal environment of oncogenesis, which consists of tumor cells, mesenchymal cells, micrangiun, microlymphatics, tissue fluids, and a small number of infiltrating cells. It also includes many soluble molecules (such as growth factors, cytokines), metabolites and extracellular matrix. With the occurrence of EMT, many nuclear substances in tumor microenvironment changed, such as E-cadherin, N-cadherin, Vimentin and so on. E-cadherin, a classical cadherin, participates in information transmission and differentiation between cells, promotes cell adhesion and aggregation and maintains the integrity and polarity of epithelial morphology and structure. Contrary to E-cadherin, N-cadherin can affect the morphology and behavior of epithelial cells, promote the adhesion between epithelial cells and mesenchymal cells, and enable cells to acquire the ability of movement and migration, which is known as "cadherin switch".25-27

As important transcription factors of EMT, Slug, Snail and Twist play a key role in the regulation of EMT by directly inhibiting the synthesis of adhesion components (such as E-cadherin and β-catenin) and

Figure 8 Effect of WLXZW on the expression of EMT-related proteins in BGC823 cell
A: expressions of EMT marker proteins (N-cadherin, Vimentin and E-cadherin) in BGC823 cell at 24 h; B: effect of WLXZW on the expressions of EMT transcription proteins (Slug, Snail and Twist) in BGC823 cell at 24 h; C,D: relative expression level of protein was expressed by measuring the gray ratio of protein band to internal reference protein band. 1: control group (blank serum); 2: TGF-β, group (TGF-β, 10 ng/mL + blank serum); 3: TGF-β1 + WLXZW low does group (TGF-β, 10 ng/mL + medicated serum of WLXZW concentration of 0.74 mg/mL); 4: TGF-β1 + WLXZW medium does group (TGF-β, 10 ng/mL + medicated serum of WLXZW concentration of 0.82 mg/mL); 5: TGF-β1 + WLXZW high does group (TGF-β, 10 ng/mL + medicated serum of WLXZW concentration of 0.91 mg/mL); 6: LY364947 group (TGF-β, 10 ng/mL + blank serum + LY364947 2 μM). WLXZW: Wulong Xiaozheng Wan; TGF-β: transforming growth factor-β; EMT: epithelial-mesenchymal transition. Compared with control group, \(^p < 0.01\); compared with TGF-β group, \(^p < 0.01\).
tight-junction components (such as Occludin and ZO1), reducing cell adhesion, and increasing cell phenotypic plasticity and motility by binding the E-box junction motif of E-cadherin promoter. In this study, we found that the expression of E-cadherin decreased significantly, and the expressions of N-cadherin, Vimentin, Slug, Snail, and Twist increased significantly after stimulation of TGF-β1, which further induced the occurrence of EMT and the changes of adhesion, invasion, and migration. WLXZW medicated serum could significantly regulate the expressions of E-cadherin, N-cadherin, Vimentin, Snail, Twist and Slug in BGC823, thus reversed EMT.

A large number of inflammatory factors existing in the tumor microenvironment could aggravate the inflammation effect by recruiting inflammatory cells in the tumor area and causing the accumulation of vasodilation. It could also directly induce the change of cell phenotype, and play the role of promoting cancer by promoting the change of proliferation, invasion, and migration. In recent years, the induction of EMT by inflammatory cytokines has become a hotspot in the field of cancer research.

TGF-β1, an important inflammatory cytokine in tumor microenvironment secreted mainly by tumor cells and stromal cells (including immune cells and fibroblasts), initiated to participate in the growth, angiogenesis, migration, and invasion of tumors by binding to receptors to start Smad and nonclassical Smad signaling pathway. The Smad signaling pathway is the main mechanism of EMT induced by TGF-β1, it can induce EMT and promote tumor metastasis by up-regulating the expression of N-cadherin and inhibiting the expression of E-cadherin. MMPs are a multifunctional family of proteins, which can induce EMT and participate in angiogenesis, tissue invasion, inflammation, and metastasis by degrading and remodeling proteins in extracellular matrix. It has been found that TGF-β could directly up-regulate the expression of MMP-2 and MMP-9, and promote angiogenesis, invasion and distal metastasis of tumors. In this study, we found that the invasion and migration ability of BGC823 cells in-

Figure 9 Effect of WLXZW on the expressions of TGF-β1/Smad signaling pathway and EMT-related mRNA in BGC823 cell
A: expressions of mRNA of TGF-β1/Smad signaling pathway in BGC823 cell at 24 h; B: expressions of N-cadherin, Vimentin, and E-cadherin mRNA in BGC823 cell at 24 h; C: expressions of Slug, Snail, and Twist mRNA in BGC823 cell at 24 h. Control group (blank serum), TGF-β1 group (TGF-β1, 10 ng/mL + blank serum), low, medium and high dose group of WLXZW (TGF-β1, 10 ng/mL + medicated serum of WLXZW concentration of 0.72, 0.82, 0.89 ng/mL respectively), LY364947 group (TGF-β1, 10 ng/mL + blank serum + LY364947 2 μM), WLXZW: Wulong Xiaozheng Wan; TGF-β1: transforming growth factor-β1; EMT: epithelial-mesenchymal transition. Compared with control group, P < 0.01; compared with TGF-β1 group, P < 0.01.
creased significantly after stimulated by TGF-β1, and the expressions of MMP-2, MMP-7 and MMP-9 increased significantly, which was consistent with previous studies. WLXZW medicated serum could significantly reduce the expressions of MMP-2, MMP-7, and MMP-9 in BGC823. At present, the treatment of targeted TGF-β signal mainly includes blocking the binding of TGF-β with its receptor by neutralizing antibody, targeting the antisense oligonucleotide chain of TGF-β and inhibitor of TGF-β receptor kinase. Clinical trials show that anti-TGF-β strategy has a good anti-tumor effect, while the associated adverse reactions need to be paid enough attention. Under these circumstances, more basic and clinical studies are needed to confirm the efficacy and safety of targeting TGF-β in cancer treatment. Our previous studies have found that WLXZW could inhibit the proliferation, migration, invasion of gastric cancer BGC823 cells, and block the activation of PI3K/AKT signaling pathway by inhibiting nuclear entry of NF-κB p65 and phosphorylation of NF-κB p65. However, most of our previous studies used WLXZW to directly stimulate tumor cells to investigate the effect of anti-tumor. This method cannot observe the effect of drug absorption and metabolism on drug composition and efficacy in vivo. Therefore, we used the WLXZW medicated serum to study the anti-tumor effect in this study, which would contribute to a more comprehensive observation of the anti-cancer pharmacological mechanism of WLXZW. At the same time, we identified the main components of WLXZW by high-performance liquid chromatography, and the results showed that the main active ingredients were Syringoside, Chlorogenic acid, Acanthoside E, Isofraxidin and Juglone, which have been shown to have significant anti-neoplastic effects.37-42

In this study, we found that BGC823 stimulated by TGF-β, exhibited significant EMT characteristics, including the significant increase of E-cadherin and the decrease of N-cadherin and Vimentin. Meanwhile, cell apoptosis and adhesion between homologous cells decreased, cell-matrix adhesion, cell proliferation, invasion, and migration increased significantly. TGF-β induces the activation of TGF-β/Smad signaling pathway by binding with TβRI, further induced the phosphorylation of Smad 2 and Smad 3 and inhibition the expression of Smad 7. It also promoted the expression of EMT transcription factors and the occurrence of EMT. The expression of TβRI decreased significantly after the action of WLXZW medicated serum, and the inhibition of the TGF-β/Smad signaling pathway increased significantly with the increase of drug concentration, which further inhibited the expression of Slug, Snail and Twist, and the occurrence of EMT.

In conclusion, WLXZW medicated serum can modulate the activation of TGF-β/Smad signal pathway by inhibiting the expression of TβRI, so as to achieve the purpose of inhibiting EMT.

REFERENCES


