XJP Inhibits Apoptosis, Invasion, EMT, and Wnt/B-Catenin Pathway in Triple-Negative Breast Cancer

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Research

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Abstract

**Background:** Triple-negative breast cancer (TNBC) progresses at a rapid pace. Chemotherapy is a major clinical application. However, resistance and metastases are key barriers to chemotherapy. Xiaojin pills (XJP) have been used clinically for treating TNBC for decades. However, the potential molecular mechanisms of the effect of XJP on breast cancer is still not understood.

**Methods:** The cell viability was analyzed using Cell Counting Kit-8 (CCK-8). Flow cytometry was used to detect apoptosis, and the migration and invasion abilities of TNBC were assessed using Transwell assay. For molecular mechanisms, the protein expression levels were determined by Western blot analysis. The expression of β-catenin in the Wnt/β-catenin pathway was detected with immunofluorescence (IF).

**Results:** XJP inhibited the viability and proliferation of the TNBC cell line *in vitro*. Flow cytometry analysis showed that apoptosis increased in both MDA-MB-231 and MDA-MB-468 cells induced by XJP. The expression of the proteins associated with invasion, for example, matrix metalloproteinase (MMP) and MMP9, was reduced. Among epithelial–mesenchymal transition markers, E-cadherin was upregulated and N-cadherin was downregulated. The apoptosis-related proteins caspase-8, caspase-3, caspase-9, and Parp were all upregulated. Additionally, XJP effectively suppressed the expression of β-catenin, which belonged to the Wnt/β-catenin pathway.

**Conclusions:** These results suggested that XJP suppressed the progression of TNBC cells by suppressing apoptosis, invasion, EMT, and Wnt/β-catenin pathway.

**Background**

Breast cancer is the most frequent cancer, accounting for 30% of female cancers (1). One report indicates that 20%-30% of patients with breast cancer can develop metastases after diagnosis and primary tumor treatment, and approximately 90% of cancer-related deaths are attributed to metastases (2, 3). Only 15%-20% of the total population of breast cancers is triple negative (4). It is characterized by its highly aggressive clinical progression due to its early age, higher metastatic potential, and worse clinical outcomes (5, 6). Advances in TNBC are rapid, and current methods of controlling disease development are very limited due to the lack of common therapeutic targets (7); chemotherapy is a standard strategy to date. However, only about one-third of patients attain a pathological complete response, while the remaining patients relapse and eventually succumb to the disease (8). Resistance to chemotherapy (9) and tumor metastases in this type of cancer disturb clinical treatment. Therefore, more effective therapies for TNBC are urgently needed.

Moreover, defects in the apoptotic pathway may make cancer cells resistant to treatment because chemotherapy and irradiation act primarily by inducing apoptosis. Resistance to apoptosis may also increase the leakage of tumor cells. Thus, resistance to apoptosis is a significant clinical issue. In multicellular organisms, many mechanisms that control tissue homeostasis are linked to apoptosis.
Apoptosis can lead to certain changes in cellular properties. These changes are activation of caspases, mitochondrial depolarization, cell volume loss, and DNA fragmentation (10). Caspase cascade plays an important role in cellular death, ensuring normal tissue renewal (11). Caspases were recruited at the death-inducing signaling complex; caspase-8 acts as “initiating” caspases (12). When caspase 8 is activated, downstream effector caspases, such as caspase-3 and caspase-9, are subsequently activated, causing the cleavage of the proteins involved in the execution of apoptosis (11). Caspase-3 is a critical effector of apoptosis and is responsible for the proteolytic cleavage of key proteins such as poly(ADP-ribose) polymerase 1 (PARP1) (13). PARPs are DNA-dependent nuclear enzymes. PARP1 is considered a suitable therapeutic target for the potential treatment of cancers (14).

Epithelial–mesenchymal transition (EMT) is a continuous process of transformation of epithelial cells into motile mesenchymal cells with invasive properties; it plays an important and necessary role in cellular development and the metastasis of breast cancer (15). A decrease in the levels of the epithelial marker E-cadherin (CDH1) and an increase in the levels of the mesenchymal marker N-cadherin are the key changes during EMT (16). CDH1 is downregulated in some malignant tumors, especially undifferentiated tumors with metastatic characteristics; however, CDH2 has the opposite expression. CDH1 has a critical role in β-catenin function and stabilization; β-catenin may disassociate from E-cadherin/β-catenin complexes and translocate to the nucleus (17). β-Catenin, the core of the canonical Wnt/β-catenin pathway, plays two roles in simple epithelia: it acts as a binding partner for adherens junction proteins, such as E-cadherin, or as a messenger in the signaling pool (18). Wnt signaling regulates various cellular functions, such as cell proliferation, differentiation, and development, and processes in disease progression, such as EMT (19). In the absence of Wnt ligand, β-catenin is usually degraded by the proteasome system, including axin, glycogen synthase kinase 3 beta (GSK3β), and casein kinase 1, while the binding of Wnt to a frizzled receptor blocks the activity of destruction complex to degrade β-catenin in the presence of Wnt ligand and so β-catenin is translocated into the nucleus (20). Additionally, the dysfunctioning of Wnt/catenin signaling promotes the proliferation of mammary and colorectal cancers (21). Meanwhile, the progression of a tumor in situ to an invasive phenotype requires an increase in tumor cell plasticity (22). Matrix metalloproteinase (MMP) families, such as MMP2 and MMP9 (23), also play essential roles in breast cancer metastasis.

Therefore, a drug that influences the apoptosis and EMT of breast cancer has a significant value in the treatment. Many Chinese herbs are applied to the treatment of breast cancer. So far, XJP is a traditional Chinese medical formula used as adjuvant therapy to treat breast and thyroid cancers (24). Rong et al. (25) proved that XJP could effectively inhibit the migration and invasion of highly metastatic breast cancer cells. They also inferred that the mechanism might be related to regulating the p38 mitogen-activated protein kinase (MAPK), Jun N-terminal Kinase (JNK) MAPK pathway and the reversal of tumor cell EMT. In this study, the function and cytotoxicity of XJP in TNBC cells were investigated and its antitumor mechanism was further explored.

Materials And Methods
Herbal extraction

XJP consisted of She Xiang (Moschus), Mu Bie Zi (Cochinchina Momordica), Cao Wu (Kusnezoff Monkshood Root), Feng Xiang Zhi (Resina Liquidambaris), Ru Xiang (Olibanum), Moyo (Myrrh), Wu Ling Zhi (Trogopterus Dung), Dang Gui (Chinese Angelica), Di Long (Phertima), and Xiang Mo (Pine-soot ink). All the herbals were purchased in the Affiliated Hospital of Shandong University of Traditional Chinese Medicine, and all of them were identified by Chuanjiang Ma, who was devoted to the research of traditional medicine. The ratio of these 10 herbs was 1:5:5:5:2.5:2.5:5:2.5:0.4. The extract of XJP was prepared by decocting the dried prescription of herbs. Briefly, raw materials of XJP formulation were mixed and crushed into small pieces. Ten-time volumes of water were added with raw components and boiled for 2 h. Then, a centrifuge (Eppendorf, Germany) was used to precipitate at 3000 rpm for 5 min, five times. The decoction was concentrated by removing a portion of water in a vacuum at 60°C. Finally, the concentration of XJP was 1 g/mL. Before analysis, the decoction was filtered through a 0.22-µm polytetrafluoroethylene filter (Millipore, MA, USA). The filtered decoction was stored at 4°C before treating cells.

Cell culture

Human breast cancer cell lines MDA-MB-468 (kindly provided by Professor Changgang Sun, The Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan, Shandong) and MDA-MB-231 (kindly provided by Professor Fukai Wang, Affiliated Cancer Hospital of Shandong First Medical University, Jinan, Shandong) were cultured in Dulbecco's modified Eagle's medium (cat. no. CM15019; Macgene, China) with 10% fetal bovine serum (FBS, cat. no. 04-001-1ACS; Biological Industries, Israel), penicillin (100 U/mL), and streptomycin (100 mg/mL) (cat. no. cc004; Macgene, China). The cells were incubated at 37°C with 5% CO_2 in a humidified atmosphere. All experiments were performed on logarithmically growing cells.

Reagents

Epirubicin (cat. no. E122334; Aladdin, China) was dissolved in dimethyl sulfoxide (cat. no. D8371; Solarbio; China). CCK-8 (cat. no. CK04) was purchased from Dojindo Molecular Technologies, Inc.. Antibodies against E-cadherin (CDH1; cat. no. 22018-1-AP), β-actin (ACTB; cat. no. 66009-1-Ig), β-catenin (CTNNB1; cat. no. 51067-2-AP), N-cadherin (CDH2; cat. no. 22018-1-AP), and caspase-3 (CASP3; cat. no 66470-2-Ig.) were purchased from Proteintech Group, Inc. Antibodies against MMP2 (cat. no. ab86607), MMP9 (cat. no. ab137867), and caspase-9 (CASP9; cat. no. ab202068) were obtained from Abcam, Inc. Caspase-8 (CASP8; cat. no.4790) and Parp (PARP1; cat. no.9532) were purchased from Cell Signaling Technology. Secondary antibodies horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin G (IgG) (H + L) (cat. no. SA00013-4) and HRP-labeled goat anti-mouse IgG (H + L) (cat. no. SA00001c1) were purchased from Proteintech Group, Inc.

Cell viability assay
The sensitivity of breast cancer cells to XJP and epirubicin was detected using the Cell Counting Kit-8 (CCK-8) following the manufacturer's protocols. Briefly, MDA-MB-231 (6.0 \times 10^3 \text{ cells/well}) and MDA-MB-468 (1.0 \times 10^4 \text{ cells/well}) cells were cultured in quintuplicate in 96-well plates at 37°C overnight and treated with the drugs at different concentrations: [epirubicin, MDA-MB-231 (0, 2.5, 5, 10, 20, and 40 \mu g/mL) and MDA-MB-468 (0, 0.0625, 0.25, 1, 4, and 16 \mu g/mL)]; [XJP, MDA-MB-231 (0, 2.5, 5, 10, 20, and 40 mg/mL), and MDA-MB-468 (0, 5, 10, 20, 40, and 80 mg/mL)] for 48 h. Subsequently, the medium was removed, and the cells were incubated with 90 \mu L of new culture medium supplemented with 10 \mu L of CCK-8 reagents for 40 min. The absorption at 450 nm was detected using a spectrophotometer (Type, 1510; Thermo Fisher Scientific Inc.).

Flow cytometric analysis

Cell apoptosis was assessed using annexin V-fluorescein isothiocyanate (FITC)/propidium iodide kit following the supplier's protocols. The cells were seeded at a density of 3 \cdot 10^5 \text{ cells/well} in six-well plates. After 24 h of culture, different concentrations of XJP [MDA-MB-231 (0, 17, and 34 mg/mL) and MDA-MB-468 (0, 5, and 10 mg/mL)] were added to the plates, and the cells were further cultured for 48 h. After incubation, the cells were collected. Then, 5 \mu L of FITC and 5 \mu L of Polyimide (PI) were added per well. The cells were then analyzed using the Fluorescence Activating Cell Sorter (FACS) Calibur system (BD Company, NJ, USA) in 15 min.

Cell migration and invasion assay

Twenty-four-well plates were used to determine the effect of drugs on the migration and invasion of breast cancer cells. Breast cancer cells were harvested and resuspended in the serum-free medium supplemented with drugs at different concentrations (XJP for MDA-MB-231 cells at 0, 3.75, and 7.5 mg/mL; and for MDA-MB-468 at 0, 2, and 4 mg/mL). An 8-\mu m-pore-size filter (cat. no. 353097; BD Biosciences, NJ, USA) was pre-coated with Matrigel (cat. no. 354234; BD Biosciences) at 37°C for 30 min (Matrigel was abandoned for migration assay), and the lower chamber was filled with 500 \mu L of the medium containing 10% FBS. Suspended breast cancer cells (5 \cdot 10^4 \text{ cells/well} in 400 \mu L of medium) were seeded in the upper chamber and incubated for 48 h at 37°C. Subsequently, the filter was fixed with 4% paraformaldehyde solution (cat. no. P0099; Beyotime Institute of Biotechnology) for 20 min and stained with 0.1% crystal violet for 30 min at room temperature. Finally, the cells and Matrigel on the upper chamber were scraped with a cotton swab, and the invasive cells on the lower surface were counted under an inverted fluorescence microscope (Vert. A1; Carl Zeiss AG). Three fields of each sample were captured at 100× magnification.

Western blot analysis

The total proteins were obtained from breast cancer cells treated with XJP for 48 h at different concentrations [MDA-MB-231 (0, 3.75, and 7.5 mg/mL for invasion and migration, and 0, 17, and 34 mg/mL for apoptosis); MDA-MB-468 (0, 2, and 4 mg/mL for invasion and migration, and 0, 5, and 10 mg/mL for apoptosis)] The cell lysates were acquired using radio-immunoprecipitation assay (RIPA) cell
lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) and centrifuged at 13,200 rpm at 4°C for 15 min. A bicinchoninic acid assay kit (cat. no. P0012S; Beyotime Institute of Biotechnology) was used to determine the protein concentration, and 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate equal amounts of proteins (30 μg protein loaded per lane). After the proteins were transferred to Polyvinylidene fluoride (PVDF) membranes (cat. no. Ipvh00010-01; Millipore, MA, USA), the membranes were blocked with 5% nonfat milk for 1 h at room temperature and incubated with primary antibodies at a 1:1000 dilution at 4°C overnight. Appropriate secondary antibodies conjugated with horseradish peroxidase at 1:5000 dilution were used to incubate membrane-bound primary antibodies at room temperature for 1 h, followed by the development of immunoblots using an enhanced chemiluminescence kit (cat. no. WBKLS0050; Merck KGaA). The densitometry analysis was performed using the ImageJ version 1.52a (Media Cybernetics Inc.).

**Immunofluorescence**

Breast cancer cells were plated in 24-well plates containing coverslips. When the cells reached 60%-70% confluence, the media were removed and the cells were fixed with 4% paraformaldehyde for 15 min. Permeabilized cells were treated with 0.1% Triton X-100 (Solarbio, China) for 10 min, followed by blocking with 3% bovine serum albumin (Hat Biotech, China) for 1 h. Then, the cells were incubated with a primary antibody against Recombinant Human Catenin beta-1 (CTNNB1) in blocking buffer at 4℃ overnight. Next, the cells were incubated with fluorescein isothiocyanate (FITC)- or tetramethylrhodamineisothiocyanate (TRITC)-conjugated secondary antibody and 4′,6-diamidino-2-phenylindole (DAPI, Solarbio, China) at room temperature for 2 h. The coverslips were sealed with the slides using fluorescence decay-resistant medium (Hat Biotech, China).

**Statistical analysis**

All the experiments were carried out in triplicate (n = 3) unless otherwise mentioned, and the data were presented as mean ± standard deviation. Statistical analysis was performed using GraphPad Prism 8.0.1 (244) software (GraphPad Software, Inc.) by one-way analysis of variance with Dunnett's post hoc test. A P value <0.05 indicated a statistically significant difference.

**Results**

**XJP inhibited the viability of breast cancer cells**

The CCK-8 assay was performed to detect viability inhibition so as to identify the effects of XJP on breast cancer cells. Epirubicin was used as a positive control. The IC_{50} values of each compound in different cells were calculated and analyzed. The results showed that XJP inhibited cell viability in a dose-dependent manner in both MDA-MB-231 and MDA-MB-468 cells (Fig. 1). IC_{50} values of MDA-MB-231 cells and MDA-MB-468 cells were 35.04 ± 2.07 mg/mL and 10.42 ± 0.31 mg/mL, respectively (Table 1) in 48 h. The inhibitory effect of XJP on breast cancer cells was similar to that of the positive control epirubicin at high drug concentration. It indicated that XJP inhibited the viability in TNBC cells.
Table 1. IC\textsubscript{50} values of XJP and epirubicin

| Drugs          | IC\textsubscript{50}     |
|----------------|--------------------------|
|                | MDA-MB-231 cells         | MDA-MB-468 cells         |
| XJP (mg/mL)    | 35.04 ± 2.07             | 10.42 ± 0.31             |
| Epirubicin (µg/mL) | 3.11 ± 0.48           | 1.89 ± 0.44             |

IC\textsubscript{50}, Half-maximal inhibitory concentration.

**XJP-induced apoptosis of TNBC cells**

MDA-MB-231 and MDA-MB-468 cells were exposed to XJP at different concentrations to explore whether the growth inhibition of TNBC cells by XJP was due to the apoptotic response, and flow cytometry was performed to assess their apoptotic rate (Fig. 2). These results suggested that XJP accelerated the apoptosis of both MDA-MB-231 and MDA-MB-468 cells. In addition, the dose-dependent manner fits on MDA-MB-231 cells only.

**XJP inhibited the expression of apoptosis-associated proteins in breast cancer cells**

The expression level of PARP1, CASP3, CASP8, and CASP9 proteins was assessed by Western blot analysis to determine whether and how cell growth inhibition was caused by XJP. The expression levels of cleaved protein levels of PARP1, CASP3, and CASP9 significantly increased in both MDA-MB-231 (Fig. 3A and 3C) and MDA-MB-468 cells (Fig. 3B and 3D). These results indicated that XJP could inhibit PARP1, CASP8, CASP3, and CASP9 protein expression to accelerate the apoptosis of breast cancer cells.

**XJP suppressed the migration and invasion of TNBC cells**

CCK-8 was used to evaluate the viability of TNBC cells following XJP treatment to determine the appropriate drug concentration. The results suggested that the concentration of XJP lower than 7.5 mg/mL and 4 mg/mL could not inhibit the viability of MDA-MB-231 (Fig. 4A) and MDA-MB-468 cells (Fig. 4B), respectively. Therefore, different concentrations of XJP were used in subsequent experiments to treat MDA-MB-231 (0, 3.75, and 7.5 mg/mL) and MDA-MB-468 cells (0, 2, and 4 mg/mL) for 48 h.

Transwell assays were performed in MDA-MB-231 and MDA-MB-468 cells to further investigate the effects of XJP on breast cancer invasion and migration. The results revealed that XJP significantly suppressed the migration of MDA-MB-231 cells (Fig. 5A and 5B, $P < 0.01$ at 3.75 mg/mL, $P < 0.001$ at 7.5 mg/mL) and MDA-MB-468 cells (Fig. 6C and 6D, $P < 0.01$ at 2 mg/mL, $P < 0.001$ at 4 mg/mL) compared with that in the control group. The effect of XJP on the invasiveness of breast cancer cells was further examined. The results demonstrated that XJP suppressed the invasive ability of MDA-MB-231 (Fig. 6A...
and 6B, \( P < 0.01 \) at 3.75 mg/mL, \( P < 0.001 \) at 7.5 mg/mL) and MDA-MB-468 (Fig. 6C and 6D, \( P < 0.001 \) at 2 and 4 mg/mL) cells compared with that in the control group (0 mg/mL). Therefore, XJP inhibited the invasive and migratory abilities of MDA-MB-231 and MDA-MB-468 cells in a dose-dependent manner.

**XJP affected the expression of EMT-associated proteins and MMPs**

Since EMT-inducing transcription factors promote cancer metastasis, the present study examined whether XJP inhibited breast cancer migration and invasion by regulating the expression of EMT-associated proteins. As markers of EMT, the expression levels of CDH1 and CDH2 were detected by Western blot analysis. The results showed that the expression of CDH1 in MDA-MB-231 (Fig. 7A) and MDA-MB-468 (Fig. 7B) cells significantly increased following XJP treatment. However, the expression of CDH2 decreased compared with that in the control group. In addition, the relative expression level of CDH1 decreased in XJP-treated cells compared with that in the untreated control group (Fig. 7C and 7D). The expression levels of gelatinases MMP2 and MMP9, which serve crucial roles in maintaining extracellular matrix homeostasis and tumor invasion, were also significantly inhibited by XJP compared with those in the control group (Fig. 7A and 7B). These results indicated that the inhibition of XJP-induced migration and invasion might occur through the suppression of EMT and MMP expression.

**XJP inhibited the Wnt/\( \beta \)-catenin pathway in TNBC cells**

A recent study reported that Wnt signaling was associated with tumor cell migration and metastasis (26). \( \beta \)-catenin is the core molecule of the canonical Wnt pathway. As a consequence of its stabilization, it is translocated to the nucleus, where it controls gene expression and regulates the activities of downstream signals (27). In this study, \( \beta \)-catenin was downregulated in XJP-treated cells. As shown in Figure 8 A and 8B, Western blot analysis indicated that the downregulation of \( \beta \)-catenin was induced by XJP in a concentration-dependent manner in MDA-MB-231 and MDA-MB-468 cells, meanwhile the quantitative analysis of IF expression proved this result also. In addition, immunofluorescence results clearly showed that \( \beta \)-catenin displayed noticeable nuclear localization in control cells. However, when cells were treated with XJP, the intracellular distribution of \( \beta \)-catenin changed notably, as was characterized by decreased intranuclear fluorescence intensity (Fig. 8D). This phenomenon only appears in MDA-MB-231 cells. In MDA-MB-468 cells, the expression of \( \beta \)-catenin is poor at the nuclear position, but the expression in cytoplasm position is downregulated after exposure to XJP. These results indicated that XJP inhibits the Wnt/\( \beta \)-catenin pathway.

**Discussion**

TNBC is associated with higher metastasis and a poorer prognosis compared with other breast cancer subtypes due to the lack of effective chemotherapeutic drugs and frequently acquired chemo-resistance (28, 29). Late diagnosis, presence of metastasis, and adverse effects of chemotherapeutic drugs bring a huge challenge in the treatment of TNBC (30).
Apoptosis of cancer cells is essential to prevent the proliferation, metastasis, and development of chemoresistance among cancers (31). In tumor therapy, apoptosis is a well-known mechanism of cell death, involving the activation of caspases (32). Caspases are key mediators in programmed cell death or apoptosis (33). Studies showed that the caspase family was involved in extrinsic and intrinsic apoptotic pathways (34–37). The extrinsic apoptotic pathway involved CASP8 activation (38). CASP9 and CASP3 are two members of a family of proteases, and primary mediators of apoptosis (39). CASP3 is mainly activated through CASP8- and CASP9-dependent apoptotic pathways (40, 41); when the CASP3 is activated, PARP1 is degraded (42). In this study, XJP decreased the viability of TNBC cells, both MDA-MB-231 and MDA-MB-468 cells, by inducing apoptotic cell death. Further, XJP significantly promoted the apoptosis of MDA-MB-231 and MDA-MB-468 cells after 48 h by the activation of CASP3, CASP8, CASP9, and PARP1. Many Chinese herbals inhibit the proliferation of breast cancer cells by regulating their apoptosis. For example, *Ganoderma lucidum* spore oil inhibits the growth of MDA-MB-231 cells and tumors in vivo, inducing apoptosis (39). However, the mechanism of XJP for TNBC cells has not been reported. The present study filled this gap, revealing that the TNBC cells treated with XJP were found to be significantly involved in signaling pathways such as apoptosis, suggesting that XJP could activate the apoptosis of TNBC cells.

In patients with TNBC, the TNBC cells had strong invasion and migration abilities, resulting in a poor prognosis (43). MMPs are major components involved in metastasis. Especially, the increased levels of MMP2 and MMP9, two important members of MMPs, were associated with cancer aggressiveness and metastasis in TNBC (44, 45). The findings of this study showed that XJP reduced the expression of MMP2 and MMP9 in both MDA-MB-231 and MDA-MB-468 cell lines, suggesting that XJP might inhibit cell invasion and migration through the suppression of MMPs in TNBC. Studies also showed that EMT was an initial step in cancer metastasis, CDH2, which is a positive regulator and a canonical marker of EMT, has a correlation with aggressive clinical phenotype in TNBC (46). Besides, it has been proved that E-cadherin promotes metastasis in diverse models of invasive ductal carcinomas. While the loss of CDH1 increased invasion, it also reduced cancer cell proliferation and survival, circulating tumor cell number, seeding of cancer cells in distant organs, and metastasis outgrowth (47). Our results further suggested that CDH2 was suppressed while CDH1 was promoted in TNBC cells in the treatment of XJP. Meanwhile, under the condition of XJP at 3.75 mg/mL and 2 mg/mL for 48 h, the migration and invasion of MDA-MB-231 and MDA-MB-468 cells were inhibited, separately. In conclusion, proved that XJP could inhibit the migration and invasion of TNBC cells, downregulate the level of MMPs, and delay the progression of EMT.

The Wnt/β-catenin signaling pathway plays an important role in cancer progression, including regulation of transformation, cell proliferation, and invasion, ultimately leading to poor prognosis of overall survival (48–51). This study indicated that XJP had the viability of inhibiting the invasion and migration of TNBC cells and suppressing the progression of EMT. In addition, treatment with XJP at different concentrations reduced the levels of β-catenin, suggesting that XJP can function as an effective inhibitor of the Wnt/β-catenin pathway in TNBC cells.
Conclusions

This study explored that XJP, a traditional Chinese formulation, has a significant value in the treatment of TNBC. The mechanism of action includes inhibiting the expression of proteins related to migration as well as activating apoptosis related-proteins of TNBC cells. Meanwhile, it may suppress or destruct the Wnt/\(\beta\)-catenin pathway, which plays an important role in EMT biological progress. The findings indicated that XJP might serve as an assistant novel drug in the treatment of TNBC.

Abbreviations

TNBC: Triple-negative breast cancer; XJP: Xiaojin pills; CCK-8: Cell Counting Kit-8; \(\beta\)-catenin: \(\beta\)-serial protein; IF: immunofluorescence; MMP: matrix metalloproteinase; PARP1: poly(ADP-ribose) polymerase 1; EMT: Epithelial–mesenchymal transition; CDH1: E-cadherin; GSK3\(\beta\): glycogen synthase kinase 3 beta; MAPK: mitogen-activated protein kinase; JNK: Jun N-terminal Kinase; ACTB: \(\beta\)-actin; CDH2: N-cadherin; CASP: caspase; FITC: fluorescein isothiocyanate; PI: Polyimide; FACS: Fluorescence Activating Cell Sorter; RIPA: radio-immunoprecipitation assay; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF: Polyvinylidene fluoride; CTNNB1: Catenin beta-1; TRITC: tetramethylrhodamineisothiocyanate; DAPI: 4',6-diamidino-2-phenylindole.

Declarations

Acknowledgments

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Authors’ contributions

DDF performed the experiments and wrote the original draft. JWL provided valuable suggestions and helped revise the manuscript. ZYL designed the experiments and helped revise the manuscript. HZC and GXS performed the statistical analysis. HYL, MDZ, ZYL, JWL, and DDF revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Availability of data and materials

We declared that materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for noncommercial purposes, without breaching participant confidentiality. The datasets generated/analyzed during the current study are available.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors consent for publication.

Competing interests

The authors declare no competing interests.

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Figures
XJP inhibited the viability of breast cancer cells. The cells treated with epirubicin were used as the positive control. The cells were exposed to drugs for 48 h: MDA-MB-231 (A and B) and MDA-MB-468 cells (C and D). Experiments were conducted in triplicates.
Figure 2

Flow cytometry after Annexin V/PI staining, showing the percentage of apoptotic TNBC cells. The percentage of apoptotic cells increased significantly with increasing doses of XJP: (A and B) MDA-MB-231 cells; (C and D) MDA-MB-468 cells. After XJP exposure, Annexin V-FITC-positive and PI-negative cells were considered as early apoptotic cells (B+); Annexin V-FITC-positive and PI-positive cells were considered as late apoptotic cells (B++); and Annexin V-FITC-negative and PI-positive cells were considered as necrotic cells (B−). The experiments were performed in triplicate. NS, Not significant. *P < 0.05, ***P < 0.001, ****P < 0.0001.
Figure 3

Effect of XJP on the expression of apoptosis-related proteins in breast cancer cells as indicated by Western blot analysis. The expression of PARP1, CASP3, CASP9, and CASP8 in breast cancer cells increased: MDA-MB-231 cells (A and C) and MDA-MB-468 cells (B and D). The experiments were performed in triplicate. *P < 0.05, ***P < 0.001, ****P < 0.0001. ACTB, β-actin; CASP3, caspase-3; CASP8, caspase-8; CASP9, caspase-9; Parp, PARP1.
Figure 4

Effects of XJP on the viability of breast cancer cells. (A) MDA-MB-231 cells; (B) MDA-MB-468 cells. Experiments were conducted in triplicate. NS, Not significant. ****P < 0.0001.
Figure 5

Effects of XJP on the migration of TNBC cells (magnification, ×100). (A and B) MDA-MB-231 cells; (C and D) MDA-MB-468 cells. Experiments were conducted in triplicates. **P < 0.01, ***P < 0.001, ****P < 0.0001.
Figure 6

Effect of XJP on the invasion of TNBC cells (magnification, ×100). (A and B) MDA-MB-231 cells; (C and D) MDA-MB-468 cells. Experiments were conducted in triplicate. **P < 0.01, ***P < 0.001.
Figure 7

The expression of EMT-associated proteins and MMPs was examined by Western blot analysis. (A) MDA-MB-231 and (B) MDA-MB-468 cells were treated with different concentrations of XJP as indicated. ACTB was used as a loading control. The relative expression of CDH1, CDH2, MMP2, and MMP9 proteins in (C) MDA-MB-231 and (D) MDA-MB-468 cells treated with XJP at different concentrations was calculated. Experiments were conducted in triplicate. ***P < 0.001, ****P < 0.0001. ACTB, β-actin; CDH1, E-cadherin; CDH2, N-cadherin; MMP, matrix metallopeptidase.
Figure 8

XJP activated the Wnt/CTNNB1 pathway in TNBC cells. (A) The protein level of CTNNB1 was detected by Western blot analysis. (B) Quantitative analysis of the CTNNB1 level described in (A) and normalized with ACTB. (C) Quantitative analysis of IF expression. Scale bar, 100 μm. (D) IF showed that CTNNB1 expression decreased in both nucleus and cytoplasm. FITC-conjugated secondary antibody staining indicates the location of CTNNB1 (red), DAPI staining indicates the location of the nucleus (blue), and the merged image indicates the nuclear location of CTNNB1. The experiments were conducted in triplicate. CTNNB1, β-catenin. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.