Research Paper

Effects of Jatrorrhizine on Proliferation, Apoptosis and Invasion of Breast Cancer Cells by Regulating Wnt/Beta-Catenin Signaling Pathway

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To explore the effects of jatrorrhizine on proliferation, apoptosis, migration and invasion of breast cancer cells *via* the regulation of Wnt/β-catenin signaling pathway. Breast cancer cell line MDA-MB-468 was treated with varying concentrations of jatrorrhizine and then the effects of varying concentrations of jatrorrhizine on apoptosis of the cell were analyzed by flow cytometry. Western blotting was performed to test the protein expression of Wnt/beta-catenin signaling pathway. The breast cancer cells treated with 0 μM jatrorrhizine, breast cancer cells treated with 5 μM showed significant down-regulation in proliferation ($p>0.05$) and the breast cancer cells treated with 20 μM jatrorrhizine obviously decreased in proliferation activity, indicating the presence of statistical difference ($p<0.05$). The breast cancer cells treated with 0 μM jatrorrhizine and those treated with 5 μM showed an increase in apoptosis rate, the breast cancer cells treated with 20 μM jatrorrhizine obviously increased in apoptosis rate, indicating the presence of statistical difference ($p<0.05$). 0 μM group, the experiment of cell invasion and migration showed that 5 μM group experienced remarkable decline in the number of invasive and migratory cells *vs.* 0 μM group ($p<0.05$); and in this respect, 20 μM group had a marked decrease *vs.* 5 μM group, showing significant difference ($p<0.05$). In contrast with 0 μM group, apoptosis rate and B-cell lymphoma 2 expression level were observed to be an obvious decline and caspase-3 expression level presented an apparent increase in 5 μM group ($p<0.05$). In contrast with 5 μM group, apoptosis rate and B-cell lymphoma 2 expression level were observed to be an obvious decline and caspase-3 expression level presented an apparent increase in 20 μM group ($p<0.05$). After 48 h of treatment with jatrorrhizine, the beta-catenin expression in breast cancer cells was down-regulated in a concentration-dependent manner and whereas those of glycogen synthase kinase-3 and E-cadherin were up-regulated in a concentration-dependent manner, but that of interstitial marker protein N-cadherin in 5 μM group and 20 μM group was remarkably lower *vs.* 0 μM group. The regulation of Wnt/beta-catenin signaling pathway following the treatment of MDA-MB-468 with jatrorrhizine can promote apoptosis, inhibit proliferation and metastasis of breast cancer cells in a dose-dependent manner.

Key words: Jatrorrhizine, Wnt/beta-catenin, breast cancer, proliferation, apoptosis, invasion

Breast cancer is the commonest cancer in women (24.2 %) and the second leading cause of women death from cancer (15.0 %)[1]. Cancer metastasis is regarded as a key factor of breast cancer-related death. According to statistics, the 5 y survival rate of patients suffering from non-metastatic breast cancer is over 85 %, compared with 28.1 % for those with metastatic breast cancer. Unfortunately, current drug therapy for breast cancer has limited effectiveness. Thus, it is crucial to explore new tactics for effectively slowing the progression of breast cancer and reducing metastasis[2]. Jatrorrhizine is a natural berberine and alkaloid. Modern pharmacological studies have demonstrated that it has biological properties like anti-diabetes, antibiosis, anti-cancer,

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anti-obesity and anti-lipidemia. It has been shown that jatrorrhizine plays an anticancer role in various types of cancers[3]. However, its effects on proliferation, apoptosis and invasion of breast cancer cells remain unclear. Epithelial-Mesenchymal Transition (EMT) is a kind of phenotypic variation in tumor metastasis, with loss of epithelial features (e.g. decreased level of E-cadherin) and formation of mesenchymal cells (e.g. increased level of N-cadherin). The Wnt signaling pathway promotes EMT and in this process, Glycogen Synthase Kinase-3 beta (GSK-3β) and β-catenin are two essential participating proteins[4,5]. Furthermore, maladjustment of the signaling pathway is closely associated with tumor development, growth and metastasis[6]. It was also demonstrated that jatrorrhizine could inhibit the proliferation and metastasis of colorectal cancer through Wnt/β-catenin signaling pathway and EMT[7]. However, the underlying mechanism of inhibiting breast cancer by jatrorrhizine has not been fully understood. Therefore, we discussed the mechanism of inhibiting proliferation and metastasis of breast cancer cells by jatrorrhizine.

MATERIALS AND METHODS

General data:

Experiment materials: Breast cancer cell line MDA-MB-468 (Feiya Biotechnology).

Major instruments and reagents: Cell incubator (Shanghai Fuze Trading Co., Ltd., Model-Thermo Heracell 150i/240i); low-temperature and high-speed centrifuge (Shanghai Lu Xiangyi Centrifuge Instrument Co. , Ltd. Model-BH1200R); biological microscope (Jingtong Life Sciences Instrument, Model-XSP-11CD); constant temperature water bath (HengAo Technology, Model-HWT-20B); Fetal Bovine Serum (FBS) (JIANGBIN BIO); penicillin-streptomycin double antibody solution (Shanghai Mingbo Biotechnology Co., Ltd); mouse anti-human lymphoid enhancer factor 1 monoclonal antibody (Shanghai Jianglai Biotechnology Co. , Ltd.); mouse anti-human CD44 monoclonal antibody (Taizei); rabbit anti-human Cyclin D1 monoclonal antibody (Shanghai Future Industry Co., Ltd.) and Jatrorrhizine (Chengdu Hurbpurify Co., Ltd, purity >98 %).

Methods:

Cells recoveries and culture: Cells were removed from liquid nitrogen and melted in a hot water bath under the temperature of 37°. In 15 ml centrifugal tube, complete medium was added in advance, evenly mixed and then centrifuged at the rate of 800 rpm for 3 min. The supernatant was discarded and 1 ml complete medium was added and evenly mixed. The liquid was transferred to a petri dish or culture plate containing complete medium and continued to be cultured in a constant temperature incubator. When the density reached about 85 %, the medium was abandoned, the cleaning was performed once with Phosphate-Buffered Saline (PBS), the cell dissociation buffer was added and the cell morphology was observed with a microscope. When most of the cells in the dish became oval, the cells were digested and neutralized with complete medium tripled the volume of the dissociation buffer. Cells were collected into 15 ml centrifugal tube and centrifuged with 800 rpm at room temperature for 5 min. And then 1 ml complete medium was added and evenly mixed with the cells for subsequent use.

Observational index:

Cell proliferation test: The cell proliferation was tested by means of Cell Counting Kit-8 (CCK-8) obtained from Beyotime. Thyroid papillary carcinoma-1 cells were inoculated with a density of 200 cells/pore on a 96-pore plate. After inoculation, 10 µl CCK-8 reagents were added to the inoculation pores at 24, 48, 72 and 96 h respectively. The absorbance at 450 nm was measured by micro plate reader.

Apoptosis experiment: Flow cytometry was employed to analyze the effects of various concentrations of jatrorrhizine on apoptosis of breast cancer cell MDA-MB-468.

Transwell migration assay: Cells in each group were cultured in serum-free medium for 1 d, they were digested with pancreatic and was re-suspended with Roswell Park Memorial Institute (RPMI)-1640 cell culture medium and then inoculated into the upper part of transwell chamber at the density of 5×10^4/pore, 500 µl medium containing 10 % FBS was added into the lower part of transwell chamber. And the cells were cultured in an incubator containing 5 % Carbon dioxide (CO_2) at 37° for 24 h and then removed from the upper chamber, immobilized with 4 % methanol and then washed 3 times with PBS for 15 min. The staining with 2 % crystal violet was carried out for 15 min and rinsed with PBS three times. Microscopically, the number of cells on the lower surface of the filter membrane was counted and the number of transmembrane cells was used to express the migratory ability of the cells.

Transwell inversion assay: 50 µl of diluted matrigel glue was carefully added into each upper transwell chamber along the edge of chamber and then the cells were coagulated in an incubator at 37° for 0.5 h and then were cultured for 48 h after inoculation. Same procedures were performed for the remainder as the transwell migration assay.

Western blot detection: The total protein was extracted *via* the addition of protein lysate to each group of cells and its concentration was measured by bicinchoninic acid, the cells were boiled with 5×Sodium Dodecyl Sulfate (SDS) gel, then isolated by electrophoresis and transferred to Polyvinylidene Fluoride (PVDF) membrane. Then the cells were blocked with 10 % skimmed milk and incubated at room temperature for 2 h, corresponding concentration of first antibody was added and then the cells were incubated at 4° overnight and washed with Tris-Buffered Saline with Tween 20 (TBST) before the addition of second antibody and incubation in room temperature for 3 h. After cleaning with TBST buffer, the relative expression levels of Wnt/β-catenin signaling pathway-associated proteins were analyzed by Enhanced Chemiluminescence (ECL) coloration and exposure imaging, with β-actin as internal control.

Statistical method::

The data were analyzed by Statistical Package for the Social Sciences (SPSS) 22.0 software package. All measurement data with normal distribution were compared and represented by ($\bar{x}\pm s$). One-way Analysis of Variance (ANOVA) was adopted for comparisons among the groups and Student-Newman-Keuls (SNK)-q test for pairwise comparisons. The statistical results were statistically significant if $p<0.05$.

RESULTS AND DISCUSSION

5 µm group showed a marked decline in cell viability *vs.* 0 µm group ($p<0.05$). 20 µm group showed a marked decline in cell viability *vs.* 5 µm group ($p<0.05$) as shown in Table 1.

5 µm group showed a marked decline in invasive cells *vs.* 0 µm group ($p<0.05$). The number of invasive cells in 20 µm group had an obvious reduction *vs.* 5 µm group ($p<0.05$) as shown in Table 2.

5 µm group showed a marked decline in migration cells *vs.* 0 µm group ($p<0.05$). The number of migration cells in 20 µm group had an obvious reduction *vs.* 5 µm group ($p<0.05$) as shown in Table 3.

In contrast with 0 µm group, apoptosis rate and B-Cell Lymphoma 2 (BCL-2) expression level were observed to be an obvious decline and caspase-3 expression level presented an apparent increase in 5 µm group ($p<0.05$). In contrast with 5 µm group, apoptosis rate and BCL-2 expression level were observed to be an obvious decline and caspase-3 expression level presented an apparent increase in 20 µm group ($p<0.05$) as shown in Table 4.

Following the treatment with jatrorrhizine for 48 h, the β-catenin expression in breast cancer cells was down-regulated in a concentration-dependent manner and whereas those of GSK-3β and E-cadherin were up-regulated in a concentration-dependent manner, but that of interstitial marker protein N-cadherin in 5 µm group and 20 µm group was remarkably lower *vs.* 0 µm group as shown in Table 5.

TABLE 1: CHANGES IN CELL VIABILITY ($\bar{x}\pm s$)

Grouping	n	Cell viability (%)	
		12 h	48 h
0 µm group	6	100±0.01	100±0.01
5 µm group	6	75.48±2.16[a]	60.67±1.73[a]
20 µm group	6	62.53±4.75[ab]	46.33±3.41[ab]
F		159.61	633.74
p		<0.001	<0.001

Note: 0 µm group, [a]$p<0.05$ and 5 µm group, [b]$p<0.05$

TABLE 2: CHANGES IN CELL INVASION ($\bar{x}\pm s$)

Grouping	n	Proportion of invasive cells (%)
0 µm group	6	98.97±1.03
5 µm group	6	50.15±3.99[a]
20 µm group	6	18.36±1.83[ab]
F		937.14
p		<0.001

Note: 0 µm group, [a]$p<0.05$ and 5 µm group, [b]$p<0.05$

TABLE 3: CHANGES IN CELL MIGRATION (x̄±s)

Grouping	n	Proportion of migration cells (%)
0 μm group	6	92.96±4.58
5 μm group	6	57.89±5.27[a]
20 μm group	6	21.53±2.15[ab]
F		286.83
p		<0.001

Note: 0 μm group, [a]p<0.05 and 5 μm group, [b]p<0.05

TABLE 4: CHANGES IN APOPTOSIS RATE (x̄±s)

Grouping	n	Apoptosis rate (%)	BCL-2	Caspase-3
0 μm group	6	0.59±0.05	1.10±0.07	0.64±0.02
5 μm group	6	3.35±0.23[a]	0.86±0.10[a]	1.07±0.17[a]
20 μm group	6	12.25±0.26[ab]	0.58±0.17[ab]	1.86±0.20[ab]
F		3622.49	18.56	66.3
p		<0.001	0.001	<0.001

Note: 0 μm group, [a]p<0.05 and 5 μm group, [b]p<0.05

TABLE 5: COMPARISON OF WNT/β-CATENIN SIGNALING PATHWAY-ASSOCIATED PROTEIN EXPRESSION IN CELLS

Grouping	n	GSK-3ß	ß-Catenin	N-cadherin	E-cadherin
0 μm group	6	1.03±0.03	1.04±0.07	1.05±0.05	1.06±0.07
5 μm group	6	1.45±0.11[a]	0.86±0.06[a]	0.81±0.10[a]	1.46±0.13[a]
20 μm group	6	1.83±0.15[ab]	0.57±0.10[ab]	0.76±0.07[a]	1.76±0.18[ab]
F		5.652	4.652	3.542	4.852
p		<0.001	<0.001	<0.001	<0.001

Note: 0 μm group, [a]p<0.05 and 5 μm group, [b]p<0.05

Jatrorrhizine has been reported to fight against Hydrogen peroxide (H_2O_2)-induced Phaeochromocytoma (PC12) cell line death and have antioxidant effects[8]. It was observed that jatrorrhizine contained lower cytotoxicity than berberine and had an anti-hypercholesterolemia effect through the up-regulation of the messenger RNA (mRNA) and protein of low-density lipoprotein receptor and constitutive expression of a cholesterol-7alpha-hydroxylase[9]. At present, the research on jatrorrhizine is more concerned about its pharmacological activity, but rare about its antitumor activity. This study investigated the effects of jatrorrhizine on the growth, apoptosis and metastasis of breast cancer cells; it found that the proliferation of breast cancer cells could be inhibited by jatrorrhizine in a concentration-dependent manner. Moreover, jatrorrhizine induced apoptosis and inhibited the migration and invasion of the cancer cells. The development and progression of cancer could be effectively inhibited in the way of promoting apoptosis[10]. Mitochondrial disorder and apoptosis in breast cancer cell MDA-MB-231 were induced by jatrorrhizine (10-30 μm). The mechanisms were connected with destruction of transmembrane potential (ΔΨm), up regulation of pro-apoptotic protein Bcl-2-associated X protein and down regulation of anti-apoptotic protein BCL-2, together with apoptosis-associated proteins like procaspase-3, procaspase-8, procaspase-9 and poly adenosine diphosphate-ribose polymerase[11]. Similar to the research results. Similarly, jatrorrhizine could destroy ΔΨm and nuclear morphology of human colorectal cancer cells HCT-116 and HT-29, suggesting that the mechanism of apoptosis may be related to mitochondrial disorder[7].

EMT is a key process of cancer metastasis, epithelial cells lose their cell polarity or cell-cell adhesion and then become mesenchymal cells after acquiring migration and invasion characteristics[12]. EMT features with cellular and molecular changes, such as down regulation of epithelial markers like E-cadherin and up regulation of mesenchymal markers like Vimentin and N-cadherin[13]. Furthermore, EMT can be triggered by activating the Wnt/β-catenin pathway, which leads to cancer cell invasion[14]. In many molecular signaling pathways relating to breast cancer cell migration, β-catenin transmits the cell signals to the nucleus through the nuclear translocation from cytoplasm, thus activating many target genes regulating EMT[15]. The enhanced migration and invasion of cancer cell,

induced by EMT, was closely associated with tumor genesis and invasion as well as distant implantation. Therefore, targeting EMT is considered a promising approach for developing anti-metastatic drugs for a wide range of cancers, including breast cancer[16]. Wnt signaling pathways play a critical role in regulating cell cycle, controlling normal and malignant epithelial cell proliferation and have become an important new target for developing anticancer drugs[17]. Wnt signaling pathways include typical and non-typical pathways. Anxin-GSK-3β-β-catenin protein complex degrades after the Wnt signaling pathway is activated, releasing β-catenin, a key cytoplasmic and nuclear mediator of typical signaling pathway, which induces the proliferation and differentiation of cells[18]. In the present study, the β-catenin expression in breast cancer cells showed a marked decrease depending on the dose, whereas GSK-3β was up regulated in HCT-116 cells and was observed to be same in HT-29 cells. The cells were treated with jatrorrhizine for 48 h. Wnt signaling pathway can regulate the expression of N-cadherin, a specific mesenchymal marker during the process of EMT and plays a critical role in the metastasis of cancer. Western blotting showed that the metastasis of breast cancer cells could be inhibited by jatrorrhizine through the up-regulation of the E-cadherin expression. Additionally, N-cadherin in breast cancer cells treated with jatrorrhizine was reduced, indicating that jatrorrhizine can reverse EMT in breast cancer cells.

Likewise, jatrorrhizine (50 μg) reduced tumor angiogenesis in BALB/C nude mice with xenografted metastatic melanoma cell C8161, probably because jatrorrhizine inhibited the expression of CDH5[19]. Serine Protein Kinase (Traf2 and Nck-Interacting Kinase (TNIK)), in which Traf2 interacts with Nck, has been recognized as a vital activator of Wnt signaling pathway in promoting the progression and invasion of tumors[20]. Targeted knockout of TNIK in the human breast cancer cell line MDA-MB-231 revealed that the Wnt/β-catenin signaling pathway and the expression of EMT key proteins could be inhibited by destroying TNIK[11]. Interestingly, jatrorrhizine could bind to and interact with TNIK well. Alkaloids effectively down-regulated the expressions of TNIK, p-TNIK, β-catenin, F-actin and N-cadherin *in vitro* and up-regulated those of GSK-3β and E-cadherin *in vitro* (MDA-MB-231 and MCF-7 cells) as well as *in vivo* (Orthotic 4T1 tumor-bearing mice). Besides, tumor volume and weight could be reduced by 5 mg/kg jatrorrhizine (5 mg/kg) through the inhibition of Wnt signaling pathway and EMT process and inhibited lung metastasis of xenografted colorectal cancer cell HCT-116 in nude mouse[7]. So it can be concluded that jatrorrhizine is expected to be targeted anti-cancer drugs for TNIK and EMT.

To sum up, the regulation of Wnt/β-catenin signaling pathway following the treatment of MDA-MB-468 with jatrorrhizine can promote apoptosis, inhibit proliferation and metastasis of breast cancer cells, it is dose-dependent.

Conflict of interests:

The authors declared no conflict of interests.

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