Effects of the JWA gene in the regulation of human breast cancer cells

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Abstract. The present study aimed to investigate whether the JWA gene can regulate the proliferation, migration and invasion of human breast cancer cells through the MAPK signaling pathway. The role of JWA in proliferation, migration, invasion and apoptosis was investigated in the MDA-MB-231 human breast cancer cell line. Following transfection with JWA-small interfering (si)RNA, the effect of JWA on apoptosis was assessed by Western blot analysis, proliferation was determined using Transwell chambers and cell migration and invasion were analyzed by transwell assay. The expression levels of extracellular signal-regulated kinase (ERK) 1/2, CSBP/RK/Mpk2 kinase (p38) and c-Jun N-terminal kinase (JNK) were detected using Western blot analysis in the siRNA and control groups. The expression of JWA in the breast cancer cells was significantly lower compared with the normal breast cells. Downregulation of JWA protein levels reduced the apoptosis and enhanced proliferation, migration and invasion of the MDA-MB-231 cells *in vitro*. The results of the Western blot analysis demonstrated that, compared with the control groups, the expression levels of phosphorylated (p-)p38 decreased significantly in the JWA siRNA group. No significant changes were observed in the expression levels of p-ERK1/2 or p-JNK.

Therefore, the JWA gene may regulate human breast cancer cells through the MAPK signaling pathway using different types of regulation.

Introduction

Breast adenocarcinoma is the second leading cause of cancer-associated mortality after lung cancer and is the most common type of malignancy diagnosed in women in China (1,2). Breast cancer is the most common type of invasive cancer in women with an incidence ranging between 0.193% in eastern Africa to 0.897% in western Europe (3).

JWA, also termed ADP-ribosylation-like factor 6 interacting protein 5, was initially cloned from human tracheal bronchial epithelial cells (4). As a tumor suppressor gene, it is widely expressed in the majority of organ tissues and cultured cells, including in melanoma, gastric cancer, hepatocellular carcinoma, esophageal squamous cell carcinoma and ovarian cancer (5-9). Furthermore, certain studies have revealed that the expression of JWA in malignant tumor tissues is lower compared with the matched non-tumor tissues (8,10). However, the expression of JWA in breast cancer remains to be fully elucidated.

The mitogen-activated protein kinase (MAPK) signaling pathway, which has been observed in several studies, is aberrantly activated in a number of tumor cells (11,12). Three distinct MAPK pathways have been defined, including extracellular signal-regulated kinase (ERK) 1/2, also termed p44/p42; c-Jun N-terminal kinase (JNK), also termed stress-activated protein kinases (SAPK) and CSBP/RK/Mpk2 kinase (p38) (13). The development of breast cancer is closely associated with the activation of the MAPK pathway. The activation of p38 MAPK, but not JNK or ERK1/2, has been observed to increase by arctigenin (ATG), a natural lignan product of *Arctium lappa* in human breast cancer MDA-MB-231 cells (14).

Previous studies have reported that the expression level of JWA, a structurally novel microtubule-associated protein, is associated with the MAPK pathway in the regulation of tumor proliferation, invasion and apoptosis *in vitro* and *in vivo* (9,15). In addition, the overexpression of JWA induces apoptosis and inhibits migration and invasion in the human esophageal

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squamous cell carcinoma (ESCC) cell lines (15). Activation of the p38 MAPK signaling pathway has been found to contribute to JWA-induced tubulin polymerization, which is involved in As_2O_3-induced apoptosis (9).

In present study, the protein levels of JWA in breast cancer tissues and normal tissues were assessed to confirm whether the expression was reduced in the tumor tissues and whether the JWA gene was associated with the MAPK pathway in inducing the progression of breast cancer. Cell proliferation, migration, invasion and apoptosis were assessed in the JWA-knockdown MDA-MB-231 cell lines. The expression levels of three major MAPK pathways following JWA-knockdown in the MDA-MB-231 cells were also assessed. The aims were to examined the association between the MAPK pathway, the JWA gene and in the development of breast cancer cells to elucidate the possible mechanism underlying the malignant process of breast cancer.

Materials and methods

Breast cancer specimens. The tumor specimens and paired normal breast tissue specimens were obtained from consenting patients (15 female patients, aged from 26-53, who underwent breast surgery) and approved by the Medical Ethics Committee of Yixing People's Hospital (Yixing, China). None of the patients had received radiotherapy or chemotherapy prior to surgery.

Cell culture. The MDA-MB-231 breast cancer cells were purchased from American Type Culture Collection (Manassas, VA, USA) and were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Zhejiang, China), 100 U/ml penicillin and 100 mg/l streptomycin (Beyotime Institute of Biotechnology, Shanghai, China). The cells were cultured in a humidified incubator containing 5% CO at 37°C.

JWA small interfering (si)RNA transfection. The JWA siRNA was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA; Cat. no, sc-60820) and siRNA transfection was performed using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA). The concentration of siRNA was 150 nm after 6 h. The cells were collected after 48 h and nonsense siRNA was used as a negative control and a blank control.

MTT assay. Cell proliferation was measured using an MTT assay. The cells were collected 6 h after transfection and seeded at $2x10^4$ cells/well in DMEM containing 10% FBS in 96-well-plates. A total of five duplicate wells were used for each group and the assay was repeated three times. After 48 h, 20 μl 5 mg/ml MTT solution in PBS was added to each well for 4 h. The absorbance of each well was determined using an Infinite F50 Microplate Reader (Tecan Group, Ltd., Männedorf, Switzerland) at a wavelength of 570 nm. According to the optical densities, proliferation curves were plotted and the proliferation of the two groups were compared prior to and following transfection.

Transwell assay. Cell migration and invasion were determined using a Costar® transwell (Corning Costar, Cambridge, MA, USA) with a pore size of 0.8 μm. Matrigel (100 μl; BD Biosciences, Franklin Lakes, NJ, USA) was added to a 24-well-Transwell chamber, the normal chamber was used for cell migration assays and the Matrigel-coated chamber was used for cell invasion assays. Cell suspension (100 μl) at a concentration of $2x10^5$/ml was added to the upper chamber and DMEM containing 10% FBS was added to the lower chamber. Following incubating for 24 h at 37°C, the cells in the upper chamber were carefully removed using a cotton swab and the cells that had traversed to the reverse side of the membrane were fixed with methanol, stained with Giemsa (Sangon, Shanghai, China) and the penetrating cells were counted under a light microscope at x200 magnification (Olympus BX41, Olympus Corporation, Tokyo, Japan).

Western blot analysis. The proteins were extracted from the MDA-MB-231 cells using radioimmunoprecipitation buffer (Beyotime Institute of Biotechnology, Jiangsu, China) 72 h after transfection. The proteins (40 μg) were separated by SDS-PAGE and then blotted onto a hybond enhanced chemilluminescence (ECL) nitrocellulose membrane (GE Healthcare Life Sciences, Shanghai, China). The membrane was subsequently blocked using 5% nonfat milk at room temperature (15-25°C) for 1.5 h and incubated at 4°C overnight (15-17 h) with a rabbit polyclonal antibody to B-cell lymphoma 2 (Bcl-2; Abcam, Cambridge, UK), a rabbit polyclonal antibody to Bcl2-associated X protein (BAX), a rabbit monoclonal antibody to mitogen-activated protein kinase (MEK), a rabbit monoclonal antibody to extracellular signal-regulated kinase (ERK) 1/2, a rabbit monoclonal antibody to p38, a rabbit monoclonal antibody to c-Jun N-terminal kinases (JNK), a rabbit monoclonal antibody to phosphorylated (p-)MEK, a rabbit monoclonal antibody to p-ERK1/2, p-p38, a rabbit monoclonal antibody to p-JNK (all from Cell Signaling Technology, Inc., Danvers, MA, USA, at a dilution of 1:1,000) and a mouse anti-human GAPDH monoclonal antibody (Beyotime Institute of Biotechnology; 1:1,000).

After 15-17 h, the membrane was washed with Tris-buffered saline prior to incubation for 2 h at room temperature with the secondary antibody of immunoglobulin G (Merck KGaA, Darmstadt, Germany), labelled with alkaline phosphatase and colored by ECL, at room temperature. The membrane was then scanned using a Hewlett-Packard Development Company, 5590 (Hewlett-Packard, Palo Alto, CA, USA) to determine the relative value of protein expression.

Statistical analysis. All data were analyzsed using SPSS 14.0 software (SPSS, Inc., Chicago, IL, USA). The results of the quantitative experiments are expressed as the mean ± standard deviation. The samples were compared using Student's t-test or one-way analysis of variance (ANOVA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression level of JWA in breast cancer tissues and normal breast tissues. JWA is widely expressed in a number of organ
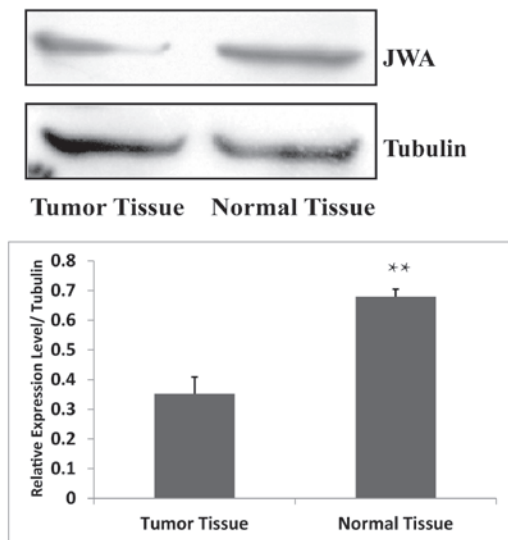
Figure 1. Different expression levels of JWA in tumor and normal tissues. JWA levels were significantly higher in the normal tissues compared with the tumor tissues (**P<0.01).
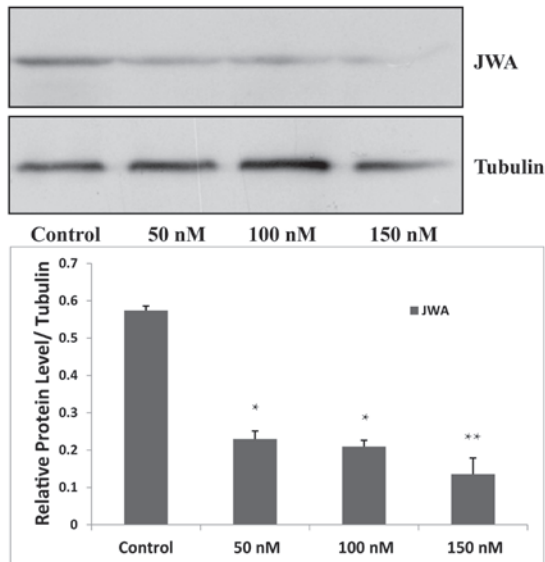

Figure 2. Optimum concentration of JWA siRNA for MDA-MB-231 cells. The protein levels of JWA were detected at various concentrations of siRNA by Western blot analysis. A concentration of 150 nM JWA siRNA reduced the protein expression levels of JWA protein significantly compared with the other concentrations (**P<0.01). A concentration of 50 nM and 100 nM reduced protein levels significantly (*P<0.05) compared with the control. siRNA, small interfering ribonucleic acid.
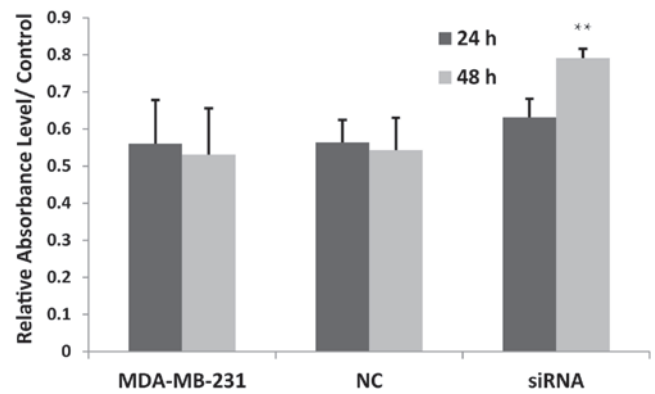

Figure 3. Proliferation of the cells was detected using an MTT assay. To determine the proliferation rate of the MDA-MB-231 cells following siRNA transfection, the cells were seeded into 96-well-plates post-transfection and an MTT assay was performed after 24 and 48 h. The proliferation rate was enhanced in siRNA treated cells compared with NC and untreated cells and was more evident 48 h after-transfection (**P<0.05). NC, normal control; siRNA, small interefereing ribonucelic acid.
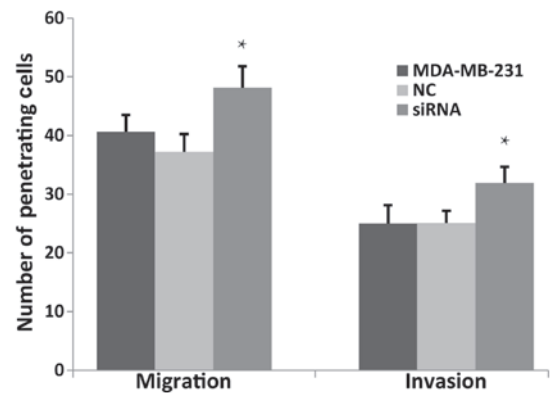

Figure 4. Levels of migration and invasion were detected using Transwell experiments. The migration and invasion capabilities increased in the siRNA transfected cells and the number of penetrating cells was significantly increased compared with the NC and untreated cell groups (*P<0.05). NC, normal control; siRNA, small interefering ribonucelic acid.

tissues and cultured cells (5-9). The present study hypothesized that JWA was also present in breast tissue. Therefore, the expression levels of JWA in breast tumor tissue and in normal breast tissue were assessed using Western blot analysis. The expression of JWA was significantly higher in the normal breast tissue compared with the breast tumor tissue (Fig. 1), indicating that the expression of JWA is low in breast cancer tissues.

siRNA downregulates the expression of JWA in MDA-MB-231 cells. In the present study, siRNA was used to knock down JWA in the MDA-MB-231 cells. siRNA targeting JWA was used at concentrations of 50, 100 and 150 nM for the siRNA group and nonsense siRNA was used for the negative control group. Untreated wild type cells were used as a blank control group. Following transfection of the MDA-MB-231 cells by siRNA, 150 nM JWA siRNA was the most effective concentration and was used for subsequent Western blot analysis (Fig. 2).

Knocking down JWA increases the proliferation rate of tumor cells. To determine the effect of JWA in breast cancer cells, the effects of siRNA on the expression of JWA were assessed by MTT assay in the MDA-MB-231 cells. The proliferation rate markedly increased compared with the negative control and blank control groups after 24 h, although these results were not statistically significant. After 48 h, the proliferation of the MDA-MB-231 cells transfected with JWA siRNA was significantly increased (Fig. 3), indicating that the JWA-knockdown upregulated the proliferation rate of breast cancer cells and that siRNA transfection had a time-dependent effect on proliferation in the MDA-MB-231 cells.
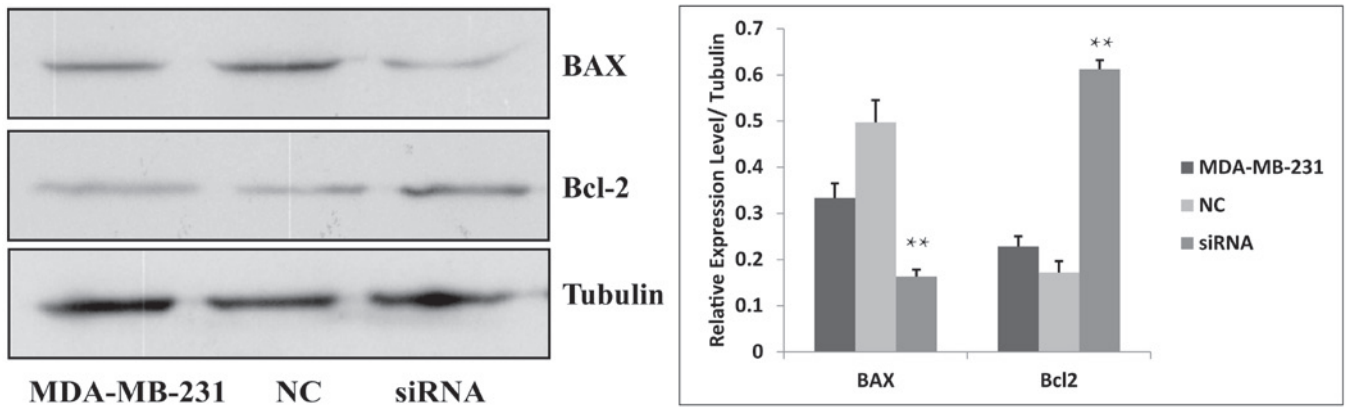
Figure 5. Protein expression levels of BAX and Bcl-2 were detected using Western blot analysis. Expression levels of BAX decreased and the levels of Bcl-2 increased significantly in the siRNA treated cells compared with the NC and untreated cell groups (**P<0.01). NC, normal control; siRNA, small interfering ribonucleic acid; Bcl-2, B-cell lymphoma 2; BAX, Bcl-2 associated X protein.
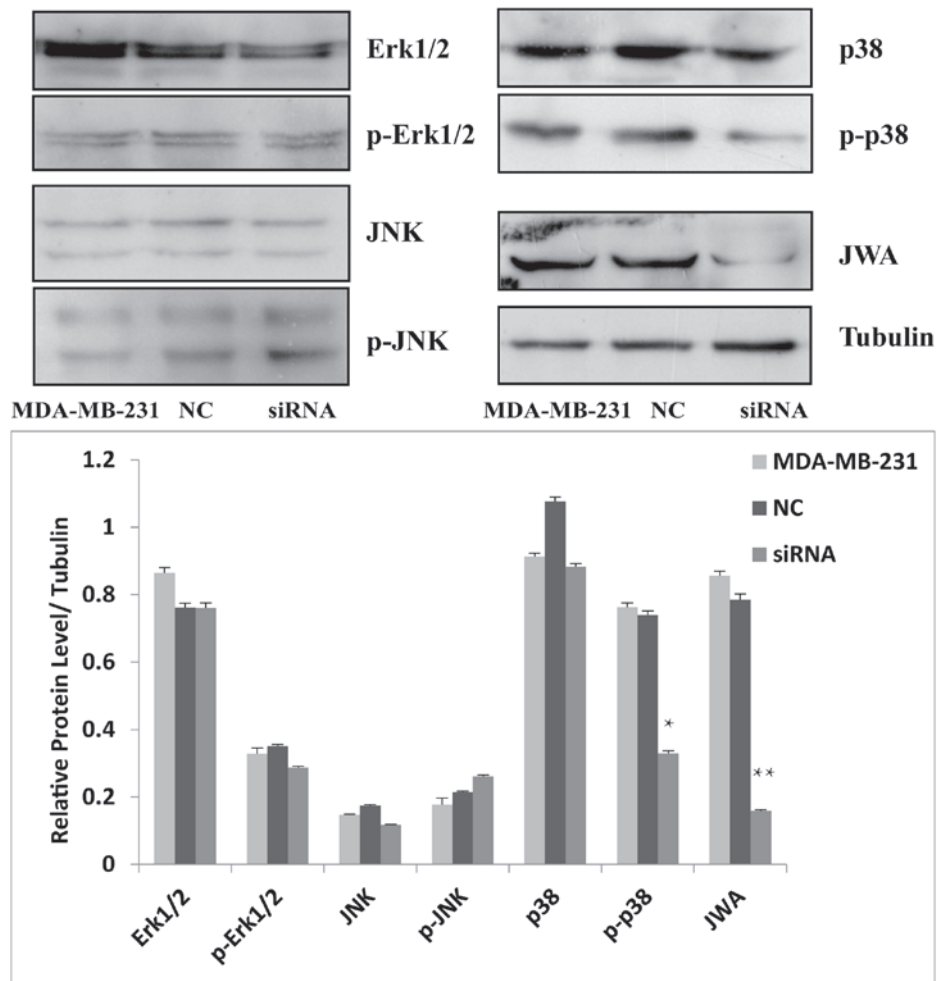

Figure 6. Protein expression levels of the MAPK pathway following JWA siRNA treatment. In the MDA-MB-231 cells, the levels of p-p38 were significantly reduced in the siRNA group compared with the NC and untreated cell groups (**P<0.05). No significant change in p-Erk1/2 or JNK was detected (P>0.05). NC, normal control, Erk1/2, extracellular signal-regulated kinases 1/2; p-Erk1/2, JNK, p-JNK, p38 or p-p38. siRNA, small interfering ribonucleic acid, p-, phosphorylated-; Erk, extracellular signal regulated kinase; JNK, c-Jun N-terminus kinase; p38, CSBP/RK/Mpk2 kinase.

JWA siRNA enhances the migration and invasion of MDA-MB-231 cells. Migration and invasion are basic biological characteristic of tumor cells. JWA is a tumor suppressor gene and knocking down JWA enhances the migration of several types of tumor cell, whereas overexpression of JWA inhibits cell migration (16). The present study aimed to measure the capability of migration and invasion of the MDA-MB-231 cells using a Transwell assay. The number of penetrating cells in the JWA siRNA group increased in the non-basement membrane chamber and the Matrigel-coated chamber (Fig. 4). These

results suggested that knock down of JWA markedly enhanced the migration and invasion of the MDA-MB-231 cells.

Knock down of JWA reduces the levels of apoptosis in tumor cells. Previous studies have indicated that JWA is important in the As$_2$O$_3$- and C/EBPα-induced apoptotic processes (17,18) and that overexpression of JWA increases the apoptosis of esophageal cancer cells (15). In this process, Bcl-2 and BAX independently regulate apoptosis by inhibiting cell death or promoting apoptosis, respectively (19). The present study demonstrated the effect of downregulating the expression of JWA on apoptosis in the MDA-MB231 cells. The protein expression of BAX was significantly decreased and the protein expression of Bcl-2 was significantly increased in the JWA siRNA group compared with the control groups (Fig. 5). These results indicated that downregulating the expression of JWA increases the apoptosis of MDA-MB-231 breast cancer cells.

p38 pathway activation following JWA-knockdown. It has been previously revealed that all trans-retinoic acid (ATRA) inhibits proliferation and induces apoptosis in Hela cells by inducing ERK phosphorylation, whereas JWA downregulation inhibits ATRA-induced ERK phosphorylation (20). To investigate the association between JWA and the MAPK pathways in breast cancer cells, the levels of phosphorylated and non-phosphorylated MAPK proteins were determined by Western blot analysis using MDA-MB-231 cells transfected with JWA siRNA. Among the three MAPK pathways, only the expression of p-p38 decreased following siRNA transfection. No changes were detected in the levels of ERK1/2, p-ERK1/2, JNK, p-JNK or p38 in response to siRNA transfection. These data revealed that JWA regulated a certain biological function in the progression of breast cancer cells via the p38 pathway (Fig. 6).

Discussion

Breast cancer is a common type of malignancy occurring worldwide and its development involves multiple factors, stages and numerous oncogenes and tumor suppressor genes alternating at the molecular level. Early systemic dissemination and local tumor progression are usually the major hallmarks of breast cancer (2). Therefore, it is vital to understand the metastais by determining the mechanisms underlying tumor progression.

Although the role of JWA has been investigated in gastric cancer, human bronchial epithelial cells, bladder cancer, human esophageal squamous cells and several other tumor cell lines (8,10,15,21,22), few studies have investigated the role of JWA in breast cancer cells. The present study investigated whether the expression levels of JWA affected the proliferation, invasion, migration and apoptosis of breast cancer cells and whether JWA regulated the biological behavior of breast cancer by activating the MAPK pathway.

Cancer cells, including breast cancer cells, are characterized by rapid proliferation rate. The results of the present study demonstrated that following the knock down of the tumor suppressor gene JWA, the proliferation rate of MDA-MB-231 cell lines increased. BAX and Bcl-2 independently regulate apoptosis, where Bcl-2 inhibits cell death

and BAX promotes apoptosis (23). In addition, the expression of Bcl-2 has a synergistic effect with a lack of BAX on apoptosis (24). Shi *et al* observed that overexpression of JWA induces apoptosis in ESCC cells (15). The present study identified that the expression of BAX decreased and the expression of Bcl-2 increased in the cells transfected with JWA siRNA. These data demonstrated that a low expression level of JWA inhibited apoptosis in breast cancer cells.

A previous study reported that JWA is associated with cytoskeletal proteins and the cell cycle (16). In ESCC cells, overexpression of JWA leads to inhibition of cell migration and invasion (15). In melanoma cells, Bai *et al* identified that invasive ability was inhibited following siRNA JWA transfection (25). This was also detected in HeLa, B16 and HCCLM3 cancer cells (16). Overexpression of JWA inhibits cell migration and the opposite effect is observed when JWA is knocked down (16). The results of the present study revealed that the migration and invasion of the cells in the siRNA JWA group were increased significantly compared with the negative and blank control groups. These results may be associated with the characteristics of JWA, associated with cytoskeletal proteins and the cell cycle (16). These findings suggested that JWA inhibited the migration and invasion of human breast cancer cells.

The MAPK signaling pathway is important in a number of the metabolic processes of tumor progression (26). Previous studies have demonstrated that a low level of JWA expression inhibits the MEK/ERK signaling pathway in MCF-7 and HeLa cells (16,18,23). The present study detected the expression levels of proteins involved in the three major pathways of the MAPK signaling pathway following JWA knockdown in the MDA-MB-231 human breast adenocarcinoma cell line. Notably, the p38 signaling pathway was inhibited following siRNA treatment, however no significant changes were observed in the MEK/ERK or JNK/SAPK signaling pathways. These findings differ from these of Ye *et al*, which revealed that a low level of JWA expression inhibited the c-Raf/MEK/ERK signaling pathway in the MCF-7 cells (23). This may be associated with the selection of different human breast adenocarcinoma cell lines. The results of the present study suggested that JWA is an important regulatory protein in the p38 signaling pathway in MDA-MB-231 cells.

In conclusion, the results of the present study led to the hypothesis that JWA regulates cell proliferation, migration, invasion and apoptosis through the p38 pathways in the MDA-MB-231 cells and that JWA has the potential to control the behavior and development of breast cancer.

Acknowledgements

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