Original Article

HMGB1 influencing invasion, migration and proliferation abilities of breast cancer cells via PI3K/AKT signaling pathway

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Abstract: Objective: To investigate the effect of HMGB1 on invasion and migration of human breast cancer cells through the PI3K/AKT signaling pathway and the related mechanism. Methods: The MCF-7 cells were selected from those breast cancer cell lines with high expression of HMGB1 by Western blotting; HMGB1 knockdown lentivirus (LV3-HMGB1) was used to silence the expression of HMGB1; efficiency of LV3-HMGB1 silencing was measured by GFP fluorescence and Western blotting; effect of silencing HMGB1 on viability of the MCF-7 cells was detected by CCK-8 assay; effect of silencing HMGB1 on the invasion ability of the MCF-7 cells was detected by Transwell invasion assay; and effect of silencing HMGB1 on the migration ability of the MCF-7 cells was detected by wound scratch assay. Cytoskeleton was stained with phalloidin to measure the invasion ability of the MCF-7 cells; the expression of proteins in the PI3K/AKT signaling pathway was detected by Western blotting. Results: LV3-HMGB1 lentivirus could effectively inhibit the expression of HMGB1; silencing the expression of HMGB1 could effectively inhibit viability of the MCF-7 cells; silencing the expression of HMGB1 could effectively inhibit the invasion, migration and proliferation abilities of the MCF-7 cells. After silencing the expression of HMGB1, morphology of the cytoskeleton changed, resulting in decreased cell motility and thus inhibiting cell migration and invasion. Silencing the expression of HMGB1 could accordingly reduce the expression of P-PI3K and P-AKT proteins in the PI3K/AKT signaling pathway. Conclusion: Silencing HMGB1 inhibits the invasion, migration and proliferation abilities of breast cancer cells by regulating the PI3K/AKT signaling pathway.

Keywords: HMGB1, breast cancer, PI3K, AKT, phalloidin

Introduction

Breast cancer is the most common malignancy in women worldwide. According to statistics of World Health Organization International Cancer Research Center, there are 1.38 million newly diagnosed patients with breast cancer across the world in 2008, accounting for 22.9% of all female cancers [1]. Statistical data show that there are 169,000 newly diagnosed patients with breast cancer in China, and it is the second most common cancer in women [2]. With progress in studies on breast cancer-related genes and its molecular mechanism, many molecules that induce cell inhibition and apoptosis have become new targets for the treatment of cancers.

High mobility group-1 protein (HMGB1) is a highly-conserved, abundant non-histone protein. It was found by Goodwin et al in bovine thymus and purified with chromosome. The molecular weight of this protein is about 30 KD; due to its low molecular mass and rich in charges, it migrates quickly in acrylamide gel electrophoresis, and thus named HMGB1 [3]. HMGB1 is a type of nuclear binding protein, which is involved in maintaining the nuclear chromatin structure, gene transcription regulation, repair, cell growth and differentiation and regulation of hormone receptor activity [4]. It has been demonstrated that HMGB1 is associated with reproductive differentiation, migration and immunoregulation of tumor cells. Current studies have shown that overexpression of genes is present in patients with gastric cancer, colorectal cancer and liver cancer, which is closely related to the invasion depth, lymph node metastasis and staging of tumors [5].
Phosphatidylinositol is a component of eukaryotic cell membrane. Phosphorylation of phosphatidylinositol by phosphoinositide kinases (PIKs) at the head part produces phosphatidylcholine 3-kinase (PI3K), which acts as a signal transduction molecule to participate in the regulation of various cellular functions. Phosphatidylinositol 3-kinase (PI3K) is a phosphatidylinositol kinase to phosphorylate the first 3-hydroxy of the inositol ring. By interacting with growth factor receptors or connexins with phosphorylated tyrosine residues, PI3K causes changes in dimer conformation and is thus activated. In addition, PI3K directly binds to p110 by the Ras protein, resulting in activation of PI3K [6]. AKT is a serine/threonine kinase, consisting of approximately 480 amino acid residues. It is highly homologous with protein kinase A (PKA) and protein kinase C (PKC), and is one of the main downstream effector molecules. The AKT family members discovered up to date fall into three subtypes: AKT1/PKBa, AKT/PKBb and AKT3/PKBg. They are encoded by three different genes, but their advanced protein structure is basically the same and they are widely expressed in various tissues [7]. The activated AKT is transferred from the cell membrane to the nucleus and the cytoplasm, to activate or inhibit its downstream target proteins by phosphorylation, and regulate proliferation, apoptosis and migration of cells.

Materials and methods

Cell line and main reagents

The human breast cancer cell line MCF-7 was purchased from Chinese Representative Culture Collection Center in Wuhan University. Cell culture conditions: cultured in DMEM containing 10% fetal bovine serum at 37°C, 5% CO₂, DMEM high glucose medium powder and fetal bovine serum (FBS) were purchased from Gibco; penicillin-streptomycin solution, dimethyl sulfoxide (DMSO) and trypsin were obtained from Sigma. The PIK3R3 rabbit monoclonal antibody was purchased from Abcam (ab186612). Transwell chambers were from Millipore (US); the Matrigel gel was from BD (US). PIK3R3 and HMGB1 silencing and control lentiviruses were purchased from Shanghai GenePharma Co., Ltd.

Cell viability detected by CCK-8 assay

Cells were digested by pancreatin and made to single cell suspension using culture medium containing serum and insulin. With concentration adjusted to 10³/L, the cells were inoculated into 96-well plates, 10 μl per well; after adherence for 24 h, the serum-free bovine insulin medium at 0.01 g/L was used instead and drug was added. There were two groups: LV3-NC control group and LV3-HMGB1 experimental group. At 24 h and 48 h after treatment, 10 μl of CCK-8 solution was added to each well, and 2 h later, the values were read from a microplate reader at 450 nm. Cell viability (%) = 100%- (OD value of the control group - OD value of the experimental group)/OD value of the control group × 100%. The experiment was performed in triplicate.

PI3K protein detection

PIK3R3 and HMGB1 silencing and control lentiviruses were purchased from Shanghai GenePharma Co., Ltd.

Migration ability of the breast cancer cells detected by Transwell invasion assay

All reagents and equipment were pre-cooled on ice. The Transwell chambers were placed in a 24-well plate. 50 μl (0.2 μg/μl) Matrigel gel was evenly applied to inner membrane of Transwell chamber, incubated for 15 min at 37°C to solidify the gel; when digested, centrifuged
and counted, the cells were diluted with 2.5 × 10⁴/mL serum-free medium to prepare cell suspension; the cell suspension was added to the upper Transwell chamber at 200 μL each well, and 400 μL of 10% FBS and medium were added to the lower Transwell chamber, placed in a 37°C incubator for culture; fixed with 4% paraformaldehyde, stained by crystal violet for 10 min, and then the cells on the inner membrane were wiped with a cotton swab, counted under a microscope, to count the cells that passed through the membrane under 4 high power fields (× 40). The experiment was performed in triplicate.

Migration ability of the breast cancer cells detected by wound scratch assay

Wound scratch assay: The MCF-7 cells were inoculated into the 6-well plate, and when cell confluence reached 90%, scratch from up to bottom using a 200 μl sterile pipette tip, observe under a microscope, to measure the initial distance of scratch (0 time); at 24 h, 48 h and 72 h, the distances of scratch were measured respectively and photographed, to calculate the cell migration rate. The experiment was performed in triplicate.

Invasion ability of the breast cancer cells detected by phalloidin staining

Cells grew on glass coverslips; 24 h later, the coverslips were fixed for 15 min in 4% paraformaldehyde, and incubated for 10 min with 0.5% Triton X-100/PBS for membrane rupture at room temperature; 1 μl of FITC-Phalloidin store solution was added into 50 μl of PBS to prepare the work solution for cytoskeleton staining. The coverslips were incubated for 40-60 min at room temperature and washed thoroughly. After extra water was removed, the coverslips were sealed with fluorescent mount-

Figure 1. Expression levels of HMGB1 in different breast cancer cell lines.

Statistical analysis

The SPSS 20.0 software was used for statistical analysis, measurement data were expressed in (X ± s), t-test was employed for comparison of means between groups, and P<0.05 indicated statistically significant difference.

Results

High expression of HMGB1 was detected in the MCF-7 cell line

In four breast cancer cell lines (BT474, SKBR3, MDA-MB-231 and MCF-7), the expression level of HMGB1 was measured by Western blotting (Figure 1). Results showed that the expression level of HMGB1 was the highest in the MCF-7 cells [(85.28 ± 7.65)%, (26.53 ± 3.34)%, (36.49 ± 4.18)% vs (11.39 ± 1.97)%, P<0.05], thus MCF-7 was selected for subsequent study.

Low expression of HMGB1 after LV3-HMGB1 was transfected into the MCF-7 cells

24 h after LV3-HMGB1 was transfected into the MCF-7 cells, it was shown under the GFP fluorescence microscope (Figure 2A) that compared with the LV3-NC group, expression of green fluorescence in the LV3-HMGB1 transfected cells was significantly increased [(89.25 ± 5.15) vs (16.52 ± 3.68), P<0.05], suggesting that LV3-HMGB1 could be well incorporated into the MCF-7 cells.

The Western blotting results (Figure 2B) showed that compared with the LV3-NC group, the HMGB1 expression level in the LV3-HMGB1 group was significantly decreased [(85.45 ± 8.29) vs (18.52 ± 6.83), P<0.05], suggesting that HMGB1 expression could be effectively reduced after LV3-HMGB1 was transfected into the MCF-7 cells.

Silencing HMGB1 inhibited the viability of the MCF-7 cells

24 h and 48 h after the MCF-7 cells were silenced with LV3-HMGB1, time-dependent da-
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Image was observed on viability of the MCF-7 cells by silencing HMGB1 (Figure 3). Compared to silencing for 24 h, inhibition was more evident at 48 h after silencing the cells with LV3-HMGB1 and cell survival rate decreased significantly [(92.26 ± 4.69)% vs (21.65 ± 3.39)%, P<0.05]. It was suggested that silencing HMGB1 had an obvious inhibitory effect on viability of the MCF-7 cells and cell survival rate decreased significantly over time.

Silencing HMGB1 inhibited the invasion ability of the MCF-7 cells

The ability of cells to pass through the Matrigel gel reflexes the invasion ability of the cells. The Transwell results (Figure 4) showed that the number of cells passing through the Matrigel gel was obviously more in the LV3-NC group than that in the LV3-HMGB1 group [(209.25 ± 7.26)% vs (49.52 ± 7.38)%], with statistically significant differences. It was indicated that silencing HMGB1 expression could effectively inhibit the invasion ability of the MCF-7 cells.

Silencing HMGB1 inhibited migration ability of the MCF-7 cells

The ability of cells to migrate can be measured by ratio of distance that the cells migrate in a...
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serum-free medium in a given time. Results of the wound scratch assay were presented in Figure 5. The width of scratches in any three parts of cells in each group was measured under a microscope, and the migration rate was calculated according to the formula: 

\[
\text{Migration rate} = \left(\frac{D_{t=24\ h,\ 48\ h,\ 72\ h} - D_{t=0\ h}}{D_{t=0\ h}}\right) \times 100\%
\]

Compared to the LV3-NC group, the migration rate was reduced significantly in the LV3-HMGB1 group [(12.26 ± 3.94)% vs (22.52 ± 6.24)%, \(P<0.05\); (39.26 ± 4.73)% vs (52.92 ± 6.74)%, \(P<0.05\); (48.26 ± 3.76)% vs (81.52 ± 9.15)%, \(P<0.05\) at 24 h, 48 h and 72 h respectively], with statistically significant difference. The results suggested that silencing HMGB1 expression could effectively inhibit the migration ability of the MCF-7 cells.

HMGB1 silencing suppressed cytoskeleton related proteins expression

Changes in the migration and invasion abilities of cells rely firstly on the changes in cell motili-
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and the changes in adhesion ability, state of the extracellular matrix and morphology will affect cell motility [8]. The morphological changes of cytoskeleton are the basis of these functional changes. Results in this study showed that silencing HMGB1 could inhibit the migration and invasion abilities of the breast cancer MCF-7 cells, so we investigated the effect of HMGB1 on the cytoskeleton morphology to speculate its mechanism. The Rho family proteins are important components of cytoskeletal proteins [9]. Rho GTP enzymes, especially RhoA, are key regulatory factors, which are mainly involved in regulation of changes in cell morphology, cell-matrix adhesion and cytoskeletal reorganization, and regulation of the invasion and metastasis processes of tumor cells [10]. We speculate that HMGB1 regulates the expression of the Rho family proteins, to activate the cytoskeleton-associated signaling pathways and regulate the migration and invasion abilities of the MCF-7 cells.

The Western blotting results (Figure 6A) showed that compared to the LV3-NC group, the expression levels of ROCK and RhoA decreased significantly in the LV3-HMGB1 group [(13.26 ± 4.24)% vs (79.52 ± 5.91)%, P<0.05; (19.66 ± 8.27)% vs (82.19 ± 11.25)%, P<0.05], suggesting silencing HMGB1 expression could down-regulate the levels of ROCK and RhoA.

The results of staining cytoskeleton with phalloidin (Figure 6B) showed that compared to the LV3-NC group, F-actin staining in the MCF-7 cells decreased remarkably, with less formation of cell membrane folds and obviously reduced number of invadopodia in the LV3-HMGB1 group.

Silencing HMGB1 inhibited expression of the PI3K/AKT signaling pathway proteins

The PI3K/AKT signaling pathway is activated in many human malignancies, including cancer of the breast, colon and rectum, ovary, pancreas and endometrium. Activation of this signaling pathway is as high as 70% in breast cancer [11]. Some studies have shown that activation of this pathway is related to characteristics of progressive breast cancer such as high histological grade, basal cell like [12] and HEB-2 subtypes [9], and also associated with poor

Figure 6. A. Expression of ROCK and RhoA detected by Western blotting after silencing HMGB1; B. Cytoskeleton staining with phalloidin in the MCF-7 cells after silencing HMGB1. Error bars represent standard error. *P<0.05.
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Figure 7. A. Expression of the proteins of the PI3K/AKT signaling pathway detected by Western blotting after silencing HMGB1. B. Statistical analysis of Western blotting assay of the PI3K/AKT signaling pathway. Error bars represent standard error. *P<0.05.

Figure 8. Ability of the MCF-7 cells to form tumor in nude mice inhibited by silencing HMGB1. Error bars represent standard error. *P<0.05.

prognosis clinically [13-15]. Therefore, we investigated whether HMGB1 gene regulation of the MCF-7 cells is related to the PIK3/AKT signaling pathway. The Western blotting results (Figure 7) showed that the expression levels of the P-PI3K and P-AKT proteins in the PIK3/AKT signaling pathway decreased accordingly after silencing HMGB1 [(21.3 ± 1.24)% vs (79.2 ± 1.86)%, (18.6 ± 2.64)% vs (83.7 ± 4.67)%, P<0.05], with statistically significant difference. The results suggested that after silencing HMGB1, changes in the migration and invasion abilities of the MCF-7 cells might be regulated through the PI3K/AKT signaling pathway.

Silencing HMGB1 inhibited growth of tumor in nude mice

Tumor formation experiment in nude mice can be used to measure the in vitro bioactivity and proliferation ability of the tumor cells. The experiment revealed that tumor formation was slower in the breast cells with HMGB1 silencing compared to the control group, and at 4 weeks, tumor volume [(0.39 ± 0.09) cm³ vs (1.58 ± 0.16) cm³, P<0.05] and mass [(0.38 ± 0.04) g vs (1.46 ± 0.12) g, Figure 8, P<0.05] were remarkably less than that in the control group, with statistically significant difference. The results suggested that after silencing HMGB1, the ability of the MCF-7 cells to form tumor in nude mice was significantly inhibited.

Discussion

Widely distributed, HMGB1 is the most abundant protein. It is located on chromosome 13q12, encoding 216 amino acid proteins [16-18]. It contains 3 domains structurally, namely A, B and C zones. Zone A is located at N terminal, zone C is located at carboxyl terminal and zone B is in the middle. C terminal is the site binding to receptors of advanced glycation end products (RAGE), which is highly conserved among species, and mammals and humans share 98% of sequence homology [19, 20]. HMGB1 is considered a carcinogenic gene related to occurrence and progression of many tumors, which enhances the proliferation ability of tumor cells and increase the cell migration and metastasis
Effect of mir on the biological behavior of hepatocellular carcinoma cells

abilities. Recent studies have demonstrated that the expression of HMGB1 increased in many human malignancies such as cancer of the breast, stomach, colon and rectum, and its expression level was related to tumor invasion and lymph node metastasis [21]. It is reported that the mean serum level was significantly higher in patients with lung cancer than that in the normal controls [25]. Its expression is increased in gastric cancer and the expression of its receptor is related to invasion and metastasis of the cancer [21]. In rectal cancer tissues, the level of HMGB1 is elevated and significantly correlated to the expression of PIK3/AKT [22]. In the rectal cancer model of rats, the size of tumor is reduced with anti-HMGB1 antibodies [23]. HMGB1 is involved in multiple links of tumor growth and metastasis and exogenously induces migration of the endothelial cells, which grow in a dose-dependent manner [24]. It protects cells from apoptosis and its level is significantly elevated in patients with colon cancer, which is correlated with the elevated expression of anti-apoptotic proteins. Inhibition of interaction with receptors can inhibit the activation of kinases. These molecular effector mechanisms are related to proliferation and invasion of tumor. In this study, by down-regulating the HMGB1 protein with lentivirus, changes were detected in the invasion and migration abilities of the breast cancer cell lines to investigate the role of HMGB in invasion and migration of the cell lines and the related mechanisms. Results showed that with decrease in the expression of HMGB1, the invasion and migration abilities of the cells reduced accordingly, suggesting that HMGB1 played an important role in invasion and metastasis of tumor.

HMGB1 expression is closely related to invasion and metastasis of gastric cancer and lung cancer [25]. Activation of p44/p42, SNP/JNK and p38 can be suppressed by inhibiting the interaction between HMGB1 and RAGE. These molecular effector mechanisms are associated with proliferation and invasion of tumor and the expression of matrix metalloproteinases (MMPs). HMGB1 activates Rac, which is considered recently a new breast cancer metastasis-related gene [26]. The HMGB1 transfected breast cancer cells up-regulates the expressions of heat shock protein 27, HMGB1 and glutathione S-transferase. The high expression of HMGB1 is closely related to poor prognosis of patients with breast cancer [27]. These results indicated that HMGB1 could regulate tumor migration and invasion through a signaling pathway, but its specific mechanism was not clear yet. Results from this study showed that compared with the LV3-NC group, F-actin staining and formation of cell membrane folds in the LV3-HMGB1 transfected MCF-7 cells was significantly reduced. Silencing the expression of HMGB1 could reduce the expression of RhoA and ROCK, indicating that HMGB1 could regulate the Rho family proteins and affect remodeling of cytoskeleton, thereby regulating migration and invasion of tumor cells.

Pathogenesis of tumor involves multi-factor and multi-step complicated pathological changes, among which, invasion is an important step in occurrence and development of tumor. A number of genes participate in growth, vascularization, invasion and metastasis of tumor. HMGB1 is a non-histone protein with highly conserved sequences, it is involved in many important biological processes such as transcription, repair, cell differentiation, growth and extracellular signal transduction, and it is also associated with proliferation, differentiation and migration of tumor cells and immune regulation etc. It plays an important part in occurrence and progression of many solid tumors, including breast cancer [28]. It has been demonstrated in a number of clinical trials that highly expressed HMGB1 is closely related to tumor invasion in many tumors [27]. However, the exact mechanism of HMGB1 for promoting tumor invasion is not clear yet. Current studies have shown that HMGB1 overexpression is closely related to the depth of tumor invasion, lymph node metastasis and tumor staging in patients with colorectal cancer and liver cancer.

The HMGB1/PI3K/AKT signaling pathway is widely present in various kinds of tumor cells. Activation of the pathway inhibits cell apoptosis, promotes progression of cell cycle, and thereby facilitates cell growth and proliferation. In addition, it is involved in tumor angiogenesis, plays an important role in tumor formation and participates in tumor invasion and metastasis [29]. The HMGB1 gene inhibits tumor formation by negatively regulating the signaling pathway and overexpression or mutation of the
gene leads to reduction or loss of inhibition on the pathway and causes cell cancerization [30]. During transduction of the PI3K/AKT signals, when activated, PIP3 as a second messenger can activate the downstream signaling molecules, and thereby result in further signal transduction, making the PI3K signaling pathway an important part in many pathophysiological processes such as differentiation, apoptosis, proliferation and migration of cells, vesicular transport, angiogenesis and malignant transformation of cells [31]. HMGB1 has phosphatase activity in normal cells, which makes PIP3 dephosphorylate to form PIP2 and lose its messenger function, thereby inhibiting the PI3K/AKT signaling pathway, leading to apoptosis and inhibiting cell proliferation [30]. In contrast, PTEN mutation or deletion causes loss of the normal inhibitory effect on transformation of PIP2 to PIP3, resulting in increased intracellular accumulation of PIP3, sustained AKT activation and the PI3K/AKT pathway in a constant state of activation, thereby inhibiting apoptosis, stimulating continuous growth of cells, promoting tumor angiogenesis, and ultimately leading to occurrence and progression of tumor [31]. It was revealed in this study that the expression of HMGB1 in breast cancer was positively correlated to that of P-PI3K and P-AKT, suggesting that abnormal activation of AKT may be closely related to the decreased expression of HMGB1; in addition, the low expression of P-PI3K and P-AKT and the decreased expression of HMGB1 jointly inhibited occurrence, progression, infiltration and metastasis of breast cancer. These findings are similar to those in other tumors such as the cancer of stomach, lung, kidney and nasopharynx and neuroglioma, etc.

In summary, it is suggested in this study that HMGB1 plays an important role in invasion and migration of breast cancer cell lines, and the relevant mechanism is further explored to measure the expression levels of HMGB1, PI3K and AKT. It allows for understanding of the clinical significance and may facilitate early diagnosis, prognosis and development of breast cancer, and HMGB1 is likely to become a protein marker for predicting the development, treatment effect and prognosis of the disease. This helps to establish justified treatment measures for the patients and provides theoretical basis for targeted therapies of breast cancer, which is of great clinical significance.

Disclosure of conflict of interest

None.

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