High-Mobility Group Box 1 (HMGB1) Promotes Angiogenesis and Tumor Migration by Regulating Hypoxia-Inducible Factor 1 (HIF-1α) Expression via the Phosphatidylinositol 3-Kinase (PI3K)/AKT Signaling Pathway in Breast Cancer Cells

Background: High-mobility group box 1 (HMGB1) is an essential contributor towards initiation and progression of many kinds of cancers. Nevertheless, our understanding of the molecular etiology of HMGB1-modulated vasculogenesis, as well as invasion, of breast cancer is poor. This study explored HMGB1 expression in breast cancer and its role in the development and spread of malignancy.

Material/Methods: We enrolled 15 patients with breast cancer who received primary surgery at the Department of Thyroid and Breast Surgery in our hospital. HMGB1 was recorded and analyzed.

Results: Our investigation successfully proves that HMGB1 is upregulated in breast cancer tissues in comparison to the surrounding non-malignant tissues. HMGB1 enhanced vessel formation in breast cancer tissues by regulating hypoxia-inducible factor 1 (HIF-1α), which in turn upregulates the expression of VEGF. Furthermore, HMGB1-mediated upregulation of HIF-1α relies on its ability to stimulate the phosphatidylinositol 3-kinase (PI3K) pathway to reinforce AKT subunit phosphorylation. HMGB1 overexpression reinforces the vasculogenesis in malignancies not only in vivo but also in vitro. Additionally, shRNA knockdown of HMGB1 prohibited the vessel-forming and invasive capabilities, downregulated VEGF and HIF-1α, and suppressed AKT phosphorylation in breast cancer cells. Most importantly, PI3K/AKT axis suppression eliminated the effect of HMGB1-modulated vascularization and invasion in breast cancer cells.

Conclusions: Our research indicates that HMGB1 serves as a crucial regulator of malignant cell-modulated vessel formation and is involved in the development of malignancy. Our findings indicate that HMGB1 is a promising target for breast cancer treatment.

MeSH Keywords: Angiogenesis Inducing Agents • Angiogenesis Inhibitors • HMGB1 Protein

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Background

Breast cancer (BC) is the most frequent cancer in women all around the world [1]. It is the most frequently diagnosed tumor and has the 6th highest malignancy-related mortality rate in Chinese females [2]. It has been previously demonstrated that metastasis is the dominant contributor to malignancy-related mortality [3]. Considerable progress has been made recently, along with emerging therapeutic approaches, including surgeries and targeted, cytotoxic, or endocrine therapies [4]. However, these approaches lack efficacy. In addition, the understanding of the molecular etiology of BC progression and development is poor [5]. Thus, it is crucial to explore innovative pathways and genes related to BC progression, along with the clinical outcome of patient therapy. This will aid in revealing the molecular etiology and developing innovative markers to diagnose BC and find targets to treat the disease.

High-mobility group box 1 protein (HMGB1) is a non-histone and ubiquitous highly conserved chromosomal protein located mainly in the stimulated chromatin, forming part of the high-mobility group proteins, which are encoded via human HMGB1 gene (13q12) [6]. It is distributed/present not only inside the cells, but also in the extracellular environment [7–10]. It is a crucial contributor to various illnesses such as sepsis, arthritis, neurodegeneration, malignancy, ischemia-reperfusion, meningitis, and aging [11–15]. The effect of HMGB1 on malignancy is complicated since it both promotes and counteracts the process of malignancy [16–18]. Suppression or enhancement of HMGB1 expression varies in different kinds of malignancies [19–21]. It is capable of displaying a wide range of expression patterns, even in a specific cancer type, as proven by a considerable inter-malignant variation of expression in different BC types [22]. Additionally, HMGB1 is released from malignant cells that are destroyed via virotherapy, radiotherapy, or chemotherapy in a pulsing manner, giving it a chance to interact with soluble components in the malignancies (e.g., microbial products, nucleic acids, or cytokines) and stimulate inflammation, adding to its complicated manner [23,24]. Nevertheless, malignant tissues slowly release HMGB1 during their development, and it consequently does not generate complexes or undergo specific modifications at the post-transcriptional level (e.g., oxidation) in order to enhance metastasis, invasion, or immune tolerance [25]. However, the biological functions and exact mechanism of HMGB1 in BC remain unclear.

In this study, we found that HMGB1 was expressed at higher levels in BC tissues compared to adjacent non-tumor tissues. Cell-line experiments were performed to explore the role of HMGB1 in BC. We found that it promotes breast cancer cell migration and tumor angiogenesis via the PI3K/AKT signaling pathway. Thus, our results indicate that HMGB1 may be a therapeutic target for BC.

Material and Methods

Participants and breast tissue specimens

Fifteen breast cancer patients who underwent primary surgery at the Department of Thyroid and Breast Surgery, Shaoxing People’s Hospital, Shaoxing Hospital of Zhejiang University were enrolled in this study. Fully informed consents were obtained from each of the participants. All procedures were approved by the Institutional Review Board of Shaoxing People’s Hospital, Shaoxing Hospital of Zhejiang University. All fresh specimens were snap-frozen with liquid nitrogen and preserved at –80°C prior to further RNA and protein experiments.

Cell cultivation and growth conditions

MCF-7 cells were acquired from Shanghai Cell Biology, Institute of the Chinese Academy of Sciences (Shanghai, People’s Republic of China). The cells were cultured using Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Grand Island, NY) and reconstituted with 10% fetal bovine serum (FBS) (Gibco) prior to incubation in a 5% CO₂ chamber at 37°C.

Cell transfections

MCF-7 cells were transfected using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific). Approximately 1×10⁵ BC cells were seeded 24 h prior to transfection. HMGB1 siRNAs were used to target HMGB1 knockdown (KD). The siRNA target sequence was: 5’-CCGGCGTTATGAAAGAGAAATGAACTCGAGTTCATTCTCTTTTCTACAGGTGGTTTTTT-3’. The shRNA lacking inhibitory effect on the expression of HMGB1 served as a negative control (NC). All shRNAs were purchased from GenePharma (Shanghai, People’s Republic of China).

Plasmids of pcDNA3.1 (vector) and HMGB1-pcDNA3.1 (HMGB1) were used for transfecting the MCF-7 cells. MCF-7 cells were seeded in 6-well cell culture plates at a cell density of 4×10⁵ cells/well. Growth medium was supplemented with 4 µg of plasmid DNA and 3 µl of lipo2000 to 24 h prior to a 6-h incubation period after transfection. The transfection admixture was then removed and replaced with normal medium, then the cells were incubated for 48 h to adapt to normal medium.

RNA isolation and qRT-PCR

Total RNA was separated using TRizol® reagent (Thermo Fisher Scientific) according to the manufacturer’s protocol. RNA specimens were preserved at –80°C. Subsequently, qRT-PCR was performed and assessed using the ABI 7500 quantitative PCR System (Applied Biosystems, Foster City, CA). Relative expression of mRNAs was evaluated according to the comparative...
cycle threshold (CT) \((2^{-\Delta\Delta CT})\) approach. GAPDH served as an internal control. Primers used for the study are listed as follows: HMGB1 forward: 5'-ATGAAGCACCAACCGTATC-3' and reverse: 5'-CTGAATTGACCTTGACTGATG-3'; GADPH forward: 5'-GTCTCCTCTGACTTCAACAGCG-3' and reverse: 5'-GTCTCCTCTGACTTCAACAGCG-3'.

**Cell migration assays**

Transwell assays were performed to examine cell migration. DMEM including 1 μg/ml mitomycin C without serum was added to a suspension of \(5 \times 10^4\) cells, which were later planted in the upper wells of poly-carbonate Transwell filters containing 24 wells (Millipore, Bedford, MA). The bottom wells contained DMEM supplemented with 10% FBS. Cells on the top were scraped off after 24-h incubation, while those in the bottom cells were fixed, stained, and quantified. Migration was evaluated in 5 random fields. Images were obtained with the help of a microscope (GX53, USA) at 20× magnification.

**Matrigel plug assay**

Male BALB/ca-nu nude mice aged 6 weeks were bought from Shanghai Experimental Animal Center and kept in aseptic conditions. MCF-7 cells were obtained and resuspended in media without serum. Aliquots of cells \((2 \times 10^6\) cells in 100 ml) were supplemented to 200 mL of \((2 \times 10^6\) cells in 100 mL). Nude mice were injected with an admixture in both flanks. Mice were killed at the 11th day after implantation. Matrigel plugs were trimmed and 50% of plugs were used for hemoglobin quantification using Drabkin's Reagent Kit (Sigma-Aldrich). The other tissues were formalin-fixed and embedded in paraffin. Tissue sections 5-µm thick were stained and evaluated using anti-CD31 antibody (Abcam).

**Western blotting (WB) analysis**

Cell lysates and tissues were homogenized with lysis buffer (Beyotime, China). Bradford assay (Bio-Rad, Hercules, CA) was performed for protein quantification. The protein samples were evaluated using standard SDS-PAGE assay. The purified/isolated proteins were separated on 8–15% Tris-HCl polyacrylamide gels (Bio-Rad) and were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The blots were incubated overnight in primary antibodies (anti-VEGF, anti-AKT, anti-HMGB1, anti-HIF, anti-p-AKT, and anti-β-actin, acquired from Cell Signaling Technology, Beverly, MA) prepared in TBST at 4°C. This was followed by incubating in secondary antibodies conjugated with horseradish peroxidase (HRP). Enhanced chemiluminescence (ECL) plus detection reagent (Pierce, Rockford, IL) was then used to assess the immunoreactive bands using the Omega 16ic Chemiluminescence Imaging System (Ultra-Lum, CA).

**Statistical analysis**

Results are represented as mean ±SEM. The 2-tailed, unequal-variance t test, or ANOVA prior to Tukey's post hoc analysis were carried out to evaluate differences among groups. Results were considered significant when the P value was less than 0.05.

**Results**

**HMGB1 was upregulated in BC**

HMGB1 expression was evaluated in 15 pairs of BC specimens and compared with surrounding non-malignant tissues via q-PCR and Western blotting. We found noticeable promotion/upregulation of HMGB1, not only at the transcriptional levels,
Figure 2. HMGB1 reinforces the BC cell migration. (A) Migration of MCF-7 cells at the bottom surface of the Transwell membranes in cells transfected with HMGB1 and vector. (B) Quantification of migrated MCF-7 cells in 5 random microscopic fields in different groups. (C) Migratory MCF-7 cells seen at the bottom of Transwell membranes in the cells transfected with negative control (NC) and si-HMGB1. (D) Quantity of migrated MCF-7 cells in 5 random microscopic fields in different groups. Results have been represented in the form of Mean ±SEM, ** p<0.01 (compared to Vector (NC) group).

Figure 3. HMGB1 reinforces malignant vessel generation. MCF-7 cells stably expressing HMGB1, si-HMGB1, or NC were supplemented with Matrigel and injected into both flanks of nude mice. Mice were executed on the 11th day subsequent to implantation, and Matrigel plugs were trimmed out. (A) Tissues were formalin-fixed and paraffin-embedded, and 5-µm sections were prepared utilizing anti-CD31 antibody; magnification, 400×. Scale bar 20 mm. (B) CD31-positive microvessels were quantified in 3 different fields per section at 400× magnification. Results are shown as mean ±SEM, n=5, * P<0.05 (compared to control group).
Figure 4. HMGB1 enhances/promotes the PI3K/AKT stimulation and HIF/VEGF expression in MCF-7 cells. (A–D) Representative immunoblots (A) as well as quantitative evaluation of AKT phosphorylation (B) HIF (C) and VEGF (D) expression in MCF-7 cells subsequent to transient transfection with HMGB1 plasmid (HMGB1) or vector alone. Results are represented as mean ±SEM. ** P<0.01 (compared to vector group).

but also at the translational level, in BC specimens in comparison to the surrounding non-malignant tissues (Figure 1A–1C).

**HMGB1 reinforced BC cell migration**

To assess the effect of HMGB1 on BC cell migration, a migration assay performed using uncoated Transwell chambers in MCF-7 cells. The migration assay showed that that HMGB1 overexpression remarkably promoted migration (Figure 2A, 2B), while the HMGB1 KD noticeably prohibited cell migration (Figure 2C, 2D). These findings suggest that HMGB1 increased the migratory ability of MCF-7 cells.

**HMGB1 enhanced vessel formation during malignancy in vivo**

To better define the contribution of HMGB1 to vessel formation in BC cells, we assessed the vessel-generating effect of HMGB1 on malignant xenografts obtained from MCF-7 cells in the mammary fat pads of NOD/SCID mice. We found that the quantity of CD31-positive microvessels was noticeably elevated by excessive miR-124 expression and was prohibited via miR-124 knock-down in MCF-7-derived xenografts in comparison to the control group (Figure 3A, 3B).

**HMGB1 increased PI3K/AKT stimulation and HIF/VEGF expression in MCF-7 cells**

PI3K/AKT axis is a crucial participant in metastasis of tumors [26]. To examine the role of HMGB1 on the PI3K/AKT axis, Western blot analysis was carried out, showing that phosphorylated AKT was promoted in MCF-7 cells via elevated HMGB1 expression in comparison to vector control (Figure 4A, 4B). This indicated that the PI3K/AKT axis participates in BC cell migration, which was modulated via HMGB1. Additionally, overexpression of HMGB1 remarkably upregulated HIF and VEGF, which are both involved in vessel formation (Figure 4C, 4D).
HMGB1 KD prohibited PI3K/AKT stimulation and HIF/VEGF expression in MCF-7 cells

To better explore the effect exerted by HMGB1 on the PI3K/AKT kinase axis, Western blot analysis was carried out, proving that phosphorylated AKT expression was inhibited by HMGB1 KD in comparison with negative control (NC) (Figure 5A, 5B). Additionally, HMGB1 KD remarkably downregulated the expression levels of HIF and VEGF (Figure 5C, 5D). The findings of our study thus indicate that HMGB1 strengthens the process of malignant vessel development through the PI3K/AKT axis.

HMGB1 modulates malignant vessel development via the PI3K/AKT/mTOR axis in BC cells

Cells were treated with AKT inhibitor MK-2206 to establish whether PI3K/AKT-modulated vessel formation relies on HMGB1. This supplementation substantially diminished the HMGB1-modulated vessel development, which supported our hypothesis (Figure 6A). Furthermore, the AKT inhibitor MK-2206 attenuated HIF and VEGF, which were upregulated by HMGB1 (Figure 6B–6D). All these results clearly indicate that HMGB1 stimulates the PI3K/AKT axis, which in turn promotes metastasis in BC cells.

Discussion

Our experiments have successf ly demonstrated that HMGB1 expression was promoted in BC tissues relative to the surrounding non-malignant tissues. HMGB1 activity was also seen in BC cells (via its overexpression) and in the knock-down assays. Our study reveals that HMGB1 enhances the migration capability and vessel production/formation in BC cells. HMGB1 enhances the PI3K/AKT stimulation, upregulating HIF and VEGF, and results in enhancement of the migratory and vessel generation capacities. Hence, our research has proven that HMGB1 has the potential to modulate biological activities in BC, and the PI3K/AKT pathway participates in this process. This research elucidates the effect of HMGB1 on BC etiology and development of therapeutic agents for its treatment.
BC is one of the most common and deadly malignancies in women, and significantly affects public health globally [27]. Recent reports have demonstrated that BC accounts for a huge fraction of malignancy-related mortality in females [28]. Consequently, there is an urgent need to identify innovative and effective treatment options for BC. However, it is critical to explore the etiology of BC progression first. During our investigation, we determined that HMGB1 expression was strongly enhanced in BC tissues in comparison with the corresponding surrounding non-malignant tissues. HMGB1, a highly conserved nuclear protein related to chromatin and damage-associated molecular pattern molecule (DAMP) outside the cells, serves as a crucial modulator of apoptosis and cell viability [29]. High HMGB1 expression is connected to all the hallmarks of malignancy, such as unlimited replicative potential, ability to form vessels, avoiding apoptosis, tolerance towards growth-suppressors, invasion, inflammation, and metastasis. In particular, HMGB1/RAGE participates in tumor metastasis by inducing matrix metalloproteinase 2 (MMP2) and MMP9 expression. The single-nucleotide polymorphism (SNP) rs243842 of the MMP2 gene was consistently associated with breast cancer prognosis in patients with disease-free survival (DFS) or overall survival (OS) events. Among DFS patients, rs243842 and rs243867 of the MMP2 gene showed statistically significant associations with a poor breast cancer prognosis, while among the OS patients, rs243842 in the MMP2 gene and rs4145277 in the HMGB1 gene were significantly associated with a poor breast cancer prognosis. In contrast, rs7656411 in TLR2 and rs7045953 in TLR4 were significantly associated with a good prognosis in the OS patients [30–33]. Our study shows that HMGB1 enhances the migration ability and vasculogenesis in BC cells. This suggests that HMGB1 may be a promising target for BC therapeutics. Inhibition of HMGB1, such as by inhibitory peptide and monoclonal antibodies, may help treat BC.

The PI3K/AKT pathway is a fundamental contributor during cellular signaling, modulation of various reactions such as proliferation, vessel generation, migration, and viability [34,35]. PI3K pathway stimulation is related to development of resistance to anti-tumor treatment, and interference with this pathway promotes the treatment response [36–38]. Many therapeutic agents aiming at 1 or more sites of the PI3K pathway have gained approval or made progress in the field of BC treatment [39–41]. Our research has clearly demonstrated that HMGB1 can modulate the PI3K/AKT kinase signaling pathway in BC cells, suggesting that HMGB1 is vital for the...
treatment of BC. Additionally, therapeutic approaches using AKT inhibitor MK-2206 nearly eliminated the increased vessel formation in BC cells triggered via excessive HMGB1 expression. These findings indicate that HMGB1 promotes migration and vessel generation in BC cells through stimulation of the PI3K/AKT axis.

**Conclusions**

Our study clearly shows the role of HMGB1 in reinforcement of vessel formation and migration of malignant tumors via regulating HIF-1α expression by the PI3K/AKT axis in BC. Our observations signify that HMGB1 is a promising candidate for diagnostic/prognostic marker and is innovative target for treating BC.

**Conflict of interests**

None.

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