Livin promotes the progression and metastasis of breast cancer through the regulation of epithelial-mesenchymal transition via the p38/GSK3β pathway

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Abstract. Epithelial-mesenchymal transition (EMT) plays a pivotal role in the invasion and metastasis of breast cancer. Livin is a recently identified member of the inhibitors of the apoptosis protein family, which has been revealed to facilitate the progression of several types of cancer. However, the role of Livin in EMT and metastasis of breast cancer and its underlying mechanisms are not fully elucidated. In the present study, the levels of Livin mRNA and protein expression were found to be elevated in breast cancer tissues and cell lines. In addition, Livin expression was positively correlated with TNM stage and lymph node metastasis in total and triple-negative breast cancer (TNBC) cases. Livin overexpression enhanced the migratory and invasive abilities of the MCF-7 cells, accompanied by increases in vimentin, N-cadherin, Snail, MMP-2 and MMP-7 and a decrease in E-cadherin. Conversely, the downregulation of Livin had the opposite effect in MDA-MB-231 cells. Furthermore, the upregulation of Livin expression clearly suppressed the activation of the p38/GSK3β pathway. In conclusion, our results revealed that Livin induced EMT through the activation of the p38/GSK3β pathway, which in turn promoted the progression and metastasis of breast cancer, especially for TNBC.

Introduction

Breast cancer is the most prevalent female cancer worldwide, and most deaths from breast cancer are due to metastasis (1,2). Triple-negative breast cancer (TNBC) which is negative for the estrogen receptor (ER), the progesterone receptor (PR) and the human epidermal growth factor receptor-2 (HER-2), presents the highest risk of recurrence and metastasis (3,4).

Studies have revealed that epithelial-mesenchymal transition (EMT) is a critical step in tumor invasion and metastasis. During the process of EMT, cancer cells lose their epithelial phenotypes and gain mesenchymal characteristics (5-7). Breast cancer cells acquire increased migratory and invasive potential through EMT (8). Our understanding of the molecular mechanisms of this process may provide effective therapeutic strategies for reducing breast cancer metastasis.

Livin is the most recently identified member of the inhibitors of the apoptosis protein (IAP) family. Numerous evidence has demonstrated that Livin is associated with a high degree of malignancy and with poor prognosis in cancer patients (9-11). Livin inhibits cell apoptosis by binding to the regulators of apoptosis and promotes cell proliferation, migration and invasion (12). A recent study revealed that Livin was involved in the regulation of EMT in colorectal cancer cells (13). However, the role of Livin in EMT and metastasis of breast cancer and its relevant mechanisms remain unclear.

In the present study, we investigated the effect of Livin on the progression and metastasis of breast cancer. Our findings demonstrated that Livin promoted invasion and metastasis in breast cancer through the regulation of EMT by activating the p38/GSK3β pathway, especially in TNBC.

Materials and methods

Tissue samples. The paraffin-embedded specimens of 150 breast cancer tissues (33 tissues of TNBC cases and 117 of non-TNBC cases) and 30 paracancerous tissues were selected from the First Affiliated Hospital of China Medical University and the Affiliated Hospital of Binzhou Medical University. The 16 pairs of primary breast cancer and the corresponding adjacent non-tumor tissues were acquired from the First Affiliated Hospital of China Medical University. The collected fresh samples were immediately stored at -80°C for protein and RNA extraction. None of the patients had undergone radiotherapy and chemotherapy prior to the sample collection. Informed consent was obtained prior to surgery, from all enrolled patients. The
study was approved by the Medical Ethics Committee of China Medical University and Binzhou Medical University.

**Cell culture.** Human breast cancer cell lines MCF-7 and MDA-MB-231, and normal human breast epithelial cell line MCF-10A were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). MCF-7 and MCF-10A cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA) and MDA-MB-231 cells were grown in Leibovitz’s L-15 medium (Gibco). Both media were supplemented with 10% fetal bovine serum (FBS; Biological Industries Israel Beit Haemek Ltd., Israel) and 1% Pen-Strep solution in a 5% CO2 humidified incubator at 37°C.

**Quantitative real-time RT-PCR.** Total RNA was extracted from the breast tissues and cells using RNAiso Plus (Takara, Dalian, China) and reverse-transcribed to synthesize cDNA using the PrimeScript® RT reagent kit (Takara) using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The quantification of Livin was determined with SYBR® Premix Ex Taq™ II (Takara) using the ΔΔCt method. The primers for Livin were 5'-GCACCGTCAAGGCTGAGAAC-3' / 5'-TCAGCGGCCAGTCATAGAAG-3'.

**Western blot analysis.** Total proteins from breast tissues and cells were extracted in RIPA buffer and quantified using the Bradford method. Lysates containing 60 µg of total proteins were subjected to 10% SDS-PAGE and subsequently transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk powder or 5% bovine serum albumin (BSA) for 2 h at room temperature. The following day, the cells were incubated with Phosphate-buffered saline (PBS). Previously identified strongly staining breast tissue sections were used as positive controls. By multiplying the staining intensity and the percentage of positive cells, we evaluated the expression of Livin. Livin was mainly located in the cytoplasm. The intensity of staining was graded as 0-3 (0, none; 1, weak; 2, moderately strong; 3, intense) and positive cell proportion was scored as 0-4 (none, 0; 1-25%, 1; 26-50%, 2; 51-75%, 3; 76-100%, 4).

**Immunofluorescence staining.** Cells were fixed with 4% paraformaldehyde and blocked with 5% BSA. The primary antibodies against E-cadherin, vimentin and N-cadherin (1:100, respectively) were incubated overnight. Secondary antibodies at room temperature for 2 h. The coverslips were incubated with DAPI for nuclear counterstaining. The results were observed using a fluorescence microscope.

**Wound healing assay.** When cell confluency reached ~90% after transfection, wounds were created in the confluent cells using a 200-µl pipette tip. The cells were rinsed with PBS to remove any free-floating cells and debris. Medium was subsequently added and culture plates were incubated at 37°C. Wound healing within the scrape lines was observed at different time-points and representative scrape lines for each cell line were photographed.

**Cell migration assay.** Cells (3x10^4) were plated in the upper Transwell chamber (Corning, Lowell, MA, USA) in 100 µl medium with 2% fetal bovine serum (FBS) after they were cultured to the exponential phase. The lower chamber was filled with 600 µl medium containing 20% FBS as a chemoattractant. After a 24-h incubation, the migrated cells were fixed with cold methanol and stained with hematoxylin. The stained migrated cells were scored and photographed under microscopic observation.

**Matrigel invasion assay.** An invasion assay was performed using the Transwell chamber precoated with diluted Matrigel (BD Biosciences, San Jose, CA, USA). Cells (1x10^5) in 100 µl medium containing 2% FBS were seeded into the upper
chamber, whereas the lower chamber was filled with 600 µl medium containing 20% FBS. The remaining experimental procedures were in accordance with the cell migration assay.

**Statistical analysis.** All statistical analyses were performed using the SPSS 16.0 statistical software (SPSS, Chicago, IL, USA). The immunohistochemistry results were analyzed using the Chi-square test and the Pearson's correlation test. Differences between groups were assessed by the Student's t-test. Data were processed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Livin is overexpressed in breast cancer tissues.** The expression of Livin in breast cancer tissues and adjacent non-tumor tissues as determined by (A) qRT-PCR and (B) western blot analysis. Both the mRNA and the protein expression levels of Livin were markedly upregulated in breast cancer tissues compared with that in paired non-tumor tissues (P<0.05).

Figure 1. Livin is overexpressed in breast cancer tissues. The expression of Livin in breast cancer tissues and adjacent non-tumor tissues as determined by (A) qRT-PCR and (B) western blot analysis. Both the mRNA and the protein expression levels of Livin were markedly upregulated in breast cancer tissues compared with that in paired non-tumor tissues (P<0.05).

**Livin is overexpressed in breast cancer cell lines.** Livin expression at the protein and mRNA levels in breast cancer cell lines MCF-7 and MDA-MB-231 and normal breast epithelial cell line MCF-10A as determined by (A) western blot analysis and (B) qRT-PCR. Livin expression at the protein and mRNA levels was significantly elevated in MDA-MB-231 and MCF-7 cells compared with those in MCF-10A cells (P<0.05).

Figure 3. Livin is overexpressed in breast cancer cells. Livin expression at the protein and mRNA levels in breast cancer cell lines MCF-7 and MDA-MB-231 and normal breast epithelial cell line MCF-10A as determined by (A) western blot analysis and (B) qRT-PCR. Livin expression at the protein and mRNA levels was significantly elevated in MDA-MB-231 and MCF-7 cells compared with those in MCF-10A cells (P<0.05).

**Livin expression is correlated with clinicopathological features of breast cancer.** We analyzed the correlation between Livin expression and the clinicopathological features of the breast cancer patients (Table I). Livin expression was significantly positively associated with TNM stage and lymph node metastasis in the breast cancer cases, especially in TNBC (P<0.01). In contrast, there was no marked correlation between Livin expression and patient age, tumor size, or that Livin was mainly located in the cytoplasm of cancer cells in breast cancer tissues (Fig. 2). The rate of positive expression of Livin in breast cancer tissues (59.3%) was significantly higher than that in adjacent non-tumor tissues (23.3%, P<0.001).

**Livin expression is correlated with clinicopathological features of breast cancer.** We analyzed the correlation between Livin expression and the clinicopathological features of the breast cancer patients (Table I). Livin expression was significantly positively associated with TNM stage and lymph node metastasis in the breast cancer cases, especially in TNBC (P<0.01). In contrast, there was no marked correlation between Livin expression and patient age, tumor size, or
Histological differentiation (P>0.05). Furthermore, the rate of Livin expression in TNBC tissues was slightly higher than that in non-TNBC tissues. However, this difference was not statistically significant. In addition, Livin expression was higher in TNBC MDA-MB-231 cells than that in non-TNBC MCF-7 cells. These results suggested that Livin expression is positively associated with the progression and metastasis of breast cancer and that Livin plays a more important role in TNBC.

Livin promotes migration and invasion in breast cancer cells. To explore whether Livin affects the migration and invasion of breast cancer cells, we upregulated Livin expression by transfection of Livin in the non-TNBC cell line MCF-7, which has low Livin expression, and knocked down Livin expression by siRNA treatment in the TNBC cell line MDA-MB-231, which exhibits high Livin expression. Transfection efficiencies were assessed by western blot analysis and qRT-PCR, respectively (Fig. 4).

Wound healing, cell migration and Matrigel invasion assays were used to investigate the effect of Livin on cell migration and invasion abilities. The upregulation of Livin led to a significant increase in the migration and invasion of the MCF-7 cells. Conversely, the migratory and invasive abilities of the MDA-MB-231 cells were suppressed by the Livin-knockdown (Fig. 5).

Livin regulates EMT in breast cancer cells. EMT is highly correlated with tumor invasion and metastasis (7,8). We therefore observed the influence of Livin on EMT in breast cancer cells. We examined the levels of several EMT-related factors using western blot analysis, after altering the expression of Livin. Livin overexpression caused a decrease in the expression of the epithelial marker E-cadherin and increases in the mesenchymal markers vimentin and N-cadherin, as well as the EMT transcription factor Snail and the metastasis-associated factors MMP-2 and MMP-7. Depletion of Livin resulted in an increase in E-cadherin and decreases in vimentin and N-cadherin as well as Snail, MMP-2 and MMP-7 (Fig. 6A). We further detected the expression of the EMT marker proteins by immunofluorescence assay and the results were consistent with those of the western blot analysis (Fig. 6B).

In addition, to determine whether Livin is associated with the EMT markers, we further performed immunohistochemical analysis of the samples from 33 cases of TNBC and 117 cases of non-TNBC (Table II). Correlation analysis revealed that Livin expression was positively correlated with the expression of vimentin and N-cadherin and negatively correlated with that of E-cadherin in TNBC. In non-TNBC, enhanced expression of Livin was significantly associated with enhanced expression of vimentin and reduced expression of E-cadherin, while Livin expression was not significantly associated with the expression of N-cadherin. These findings suggested that Livin induced EMT to promote cell migration and invasion in breast cancer cells.

The p38/GSK3β pathway is involved in the effect of Livin on EMT in breast cancer cells. Recent studies have revealed that the p38 and GSK3β pathways regulate EMT to promote progression and metastasis in several types of cancer (16,17), activating p38

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
<th>Livin (total)</th>
<th>Livin (TNBC)</th>
<th>Livin (non-TNBC)</th>
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<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
<td>P-value</td>
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<tr>
<td>Age (years) ≤45</td>
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<td>42</td>
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<tr>
<td>&gt;45</td>
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<td>47</td>
<td>0.304</td>
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<tr>
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<td>27</td>
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<tr>
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<td>42</td>
<td>0.304</td>
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<tr>
<td>&gt;5</td>
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</tr>
<tr>
<td>II</td>
<td>28</td>
<td>45</td>
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</tr>
<tr>
<td>III</td>
<td>13</td>
<td>29</td>
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<tr>
<td>TNM stage I</td>
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<tr>
<td>II</td>
<td>26</td>
<td>49</td>
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<tr>
<td>III</td>
<td>9</td>
<td>24</td>
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<tr>
<td>Positive</td>
<td>22</td>
<td>58</td>
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</tr>
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TNBC, triple-negative breast cancer.
Figure 4. Transfection efficiency of Livin identified by western blot analysis and qRT-PCR. The expression of Livin protein and mRNA in MCF-7 cells transfected with Livin overexpression plasmids and control plasmids was detected by (A) western blot analysis and (B) qRT-PCR. The expression of Livin in MDA-MB-231 cells treated with siRNA against Livin and control siRNA was examined by (C) western blot analysis and (D) qRT-PCR.

Figure 5. Change in Livin expression alters the migration and invasion of breast cancer cells. (A) Wound healing assay of cell migration. Scrape lines of each cell line of altered Livin expressions were photographed. (B) Migratory and invasive properties of cells as analyzed by cell migration and Matrigel invasion assay. *P<0.05, compared with control groups.
and inhibiting GSK3β to promote EMT. To explore the possible mechanism of Livin-induced EMT in breast cancer cells, we examined the expression of p38/GSK3β-associated factors. The overexpression of Livin in MCF-7 cells markedly enhanced the phosphorylation of p38, ATF2 and GSK3β. In contrast, the knockdown of Livin in MDA-MB-231 cells clearly inhibited the phosphorylation of p38, ATF2 and GSK3β (Fig. 7A).

Moreover, we ascertained the above results using a specific inhibitor of p38, SB203580 (Cell Signaling Technology) and an effective inhibitor of GSK3β, SB216763 (Cell Signaling Technology). SB203580 is a specific inhibitor of p38 that inhibits p38 catalytic activity by binding to the ATP binding pocket, but it does not inhibit phosphorylation of p38. SB216763 is an effective inhibitor of the GSK3β activity. Inactivation of GSK3β results in an increase in phosphorylated GSK3β. GSK3β is a downstream target of p38 and inhibiting p38 downregulates phosphorylated GSK3β expression and enhances GSK3β activity. The increased
expression of p-ATF2, p-GSK3β, vimentin and N-cadherin was eliminated and the expression of E-cadherin was partially restored by SB203580 (Fig. 7B). When SB216763 was added to the medium after Livin was upregulated, the expression of p-GSK3β, vimentin and N-cadherin was further increased and E-cadherin expression was markedly reduced (Fig. 7C). These results indicated that Livin may activate the p38/GSK3β pathway to promote EMT in breast cancer cells.

Discussion

Livin has been recently recognized as a vital molecule that participates in the development and progression of malignant tumors, such as gastric (18), colorectal (19), prostate (20) and lung cancer (21). In the present study, our results revealed that Livin expression was increased in breast cancer tissues and cells. The expression of Livin was positively correlated with the TNM stage and lymph node metastasis in breast cancer, particularly in TNBC. Moreover, the overexpression of Livin enhanced the migratory and invasive abilities of the non-TNBC cell line MCF-7 and the knockdown of Livin had the opposite effect in TNBC. These results revealed that Livin has the potential to facilitate the progression and metastasis of breast cancer, especially in TNBC, indicating it may be a therapeutic target for breast cancer treatment.
EMT is highly correlated with tumor development and progression. Through EMT, cancer cells break down the basement membrane (BM) and extracellular matrix (ECM), invading the blood or lymph vessels and spreading to other tissues and organs (22,23). During the process of EMT, the expression of markers of the epithelial phenotype, such as E-cadherin, decreases, while the expression of markers of the mesenchymal phenotype, such as vimentin and N-cadherin, increases (24-26). Several transcription factors (such as Snail and Slug) bind to the promoter regions of genes related to cell-cell adhesion and inhibit their transcription, which is the critical step in EMT (27-29). Additionally, EMT also leads to the reorganization of the ECM and EMT-inducing factors increase the expression of ECM proteins and proteases (such as MMPs) (30,31). Therefore, although the potential mechanisms involved in EMT in breast cancer are not entirely clear, we can assess EMT initiation through the detection of these factors.

According to a recent study by Ge et al (13), Livin potenti- ated the migration and invasion of colorectal cancer cells by regulating EMT. Our study revealed that Livin overexpression upregulated Snail, vimentin, N-cadherin, MMP-2 and MMP-7 and downregulated E-cadherin. In contrast, Livin knockdown had the opposite effect. Further immunohistochemistry results also established that Livin expression was positively associated with EMT in breast cancer. These results demonstrated that the influence of Livin on breast cancer aggressiveness was correlated with the induction of EMT. A wide variety of signaling pathways is implicated in the EMT process. The p38 pathway has been found to play an active role in the regulation of EMT (16,32). Recently, Ou et al (33) revealed that the knockdown of Livin suppressed the inva- sion of gastric cancer cells by inhibiting the phosphorylation of p38. GSK3β has been reported as a downstream target of p38 (34) and as also a regulator of the EMT process (17,35). Our results demonstrated that Livin expression markedly increased the phosphorylation of the p38 and GSK3β signaling proteins, revealing the activation of the p38/GSK3β pathway in Livin-overexpressing breast cancer cells. In addition, the upregu-lation of vimentin and N-cadherin expression was eliminated and E-cadherin expression was restored after the treatment of Livin-transfected cells with the inhibitor of p38. Similarly, vimentin and N-cadherin were further elevated and E-cadherin expression was markedly decreased by the inhibitor of GSK3β. Our findings revealed that Livin overexpression resulted in the activation of p38, which induced the phosphorylation of GSK3β and inhibited the GSK3β activity, ultimately contributing to the initiation of EMT. This observation revealed that Livin promoted EMT in breast cancer cells, at least partially, through the activation of the p38/GSK3β pathway. However, the specific mechanism through which Livin affects this pathway remains unknown and requires further investigation. The present study is the first to demonstrate that Livin-induced EMT is, at least partially, regulated by the p38/GSK3β signaling pathway in breast cancer. Yet, it is also noteworthy that Livin regulates EMT in breast cancer through the activation of AKT signaling (36). Whether the AKT and p38/GSK3β signaling pathways involve crosstalk or whether they are independent of each other in Livin-induced EMT of breast cancer requires further investigation. In addition, other Livin-mediated pathways should be investigated to determine whether there are other links between Livin and EMT in breast cancer.

Collectively, our results indicated that Livin promoted the progression and metastasis of breast cancer through the regulation of EMT by activating the p38/GSK3β pathway. A deeper understanding of the role of Livin-induced EMT in breast cancer may provide effective targets for breast cancer therapy, especially in TNBC.

References


