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Livin promotes progression of breast cancer through induction of epithelial–mesenchymal transition and activation of AKT signaling

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ABSTRACT

The inhibitor of apoptosis proteins (IAP) are closely correlated with proliferation, apoptosis, motility, and metastasis. Livin is the most recently identified IAP, and its role in breast progression remains unknown. In our study, analyses of 50 patients with breast cancer revealed that the positive expression rate of Livin was higher in breast cancer tissues (62%) relative to that in adjacent (35%) and normal tissues (25%). Livin expression in breast cancer correlated with the clinical stage and axillary lymph node metastasis and could be used as a prognostic marker. Our in vitro experiment revealed that Livin was highly expressed in high-invasive MDA-MB-231 cells as compared to low-invasive cells (MCF-7). Suppression of Livin by short-hairpin RNA reduced the Livin expression of MDA-MB-231 cells and subsequently inhibited tumor cell growth, proliferation, and colony formation and induced tumor cell apoptosis, motility, migration, and invasion. Overexpression of Livin in MCF7 cells resulted in increased migration and invasion capabilities of the cells without affecting proliferation and apoptosis. In addition, epithelial–mesenchymal transition (EMT) was induced by Livin expression in breast cancer cell lines. The high level of phosphorylated AKT in MDA-MB-231 cells was suppressed by Livin knockdown. Further, Livin-induced migration and invasion could be abolished by either the application of the phosphoinositide-3-kinase inhibitor LY294002 or knockdown of AKT expression using small-interfering RNA. In conclusion, Livin serves as an independent prognostic indicator for breast cancer. Livin expression promotes breast cancer metastasis through the activation of AKT signaling and induction of EMT in breast cancer cells both in vitro and in vivo.

1. Introduction

Malignant tumor progression consists of distinct steps, including tumor growth, angiogenesis, tumor cell detachment, epithelial–mesenchymal transition (EMT), growth of the macrometastasis, and so on [1]. Metastasis is generally associated with progression of malignancy, and metastatic disease accounts for 90% of deaths from all cancers [2]. However, the molecular mechanisms involved in the establishment of metastasis are largely unknown. Understanding the molecular mechanisms involved in the metastatic process could enable the identification of novel potential targets for development of more effective therapeutic interventions against established metastatic disease [3]. To this end, human non-invasive (MCF-7) and invasive (MDA-MB-231) breast cancer cell lines have been used to explore the cellular and molecular mechanisms underlying breast cancer metastasis [4].

The inhibitor of apoptosis protein (IAP) family proteins are frequently suggested as cancer therapeutic targets, and some IAP inhibitors have progressed to preclinical trials [5–8]. Livin, a novel member of the IAP family, is undetectable in most normal differentiated tissues but shows a high level of expression in a wide variety of human malignancies [6]. Elevated Livin activity is associated with poor prognosis in cancer patients [9–15]. In our study, we demonstrated that Livin is highly expressed in high-invasive breast cancer cells and breast cancer tissues, which is clinically correlated with breast tumors. To search the mechanism, we construct Livin-small hairpin RNA (shRNA) effectively downregulated Livin expression, reduced tumor cell proliferation and colony formation, induced tumor cell apoptosis in vitro, and inhibited tumorigenesis in vivo. In addition, Livin gene silencing induced G0/G1-phase cell cycle arrest. However, Livin-pcDNA3.1 elevated Livin expression and resulted in few differences in cellular proliferation, colony formation, apoptosis, and the cell cycle relative to controls.

Studies have shown that IAPs can regulate tumor cell invasion and metastasis. Livin is the most recently identified IAP that might regulate tumor cell invasion in prostate cancer directly. Livin might be

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an ideal candidate for preventing tumor cell invasion [16], although its role in breast cancer is unclear. Tumor metastasis involves a series of biological steps during which the tumor cells acquire the ability to invade surrounding tissues and survive outside the original tumor site. During the early stages, the cancer cells undergo EMT. EMT is a transdifferentiation process characterized by coordinated molecular and cellular changes that result in reduced cell–cell adhesion and a loss of apical–basolateral polarity [17,18]. EMT also induces increased motility and invasion of breast cancer cells [19,20]. Accumulated evidence has demonstrated a significant role for the AKT pathway in the development and progression of human breast cancer. AKT activation regulates survival, proliferation, the cell cycle [21], migration, and invasion [22,23] in breast cancer cells. It is unknown whether Livin can also induce AKT activation and further promote metastasis in breast cancer.

Here, we describe an alternate role for Livin in tumor progression, namely, its promotion of cellular motility. Livin is highly expressed in high-invasive breast cancer cells and is capable of promoting breast cancer cell migration, invasion via the activation of AKT signaling and the induction of EMT in vitro and in vivo.

2. Materials and methods

2.1. Cell lines and human breast tissues

A panel of breast cancer cell lines (BT549, MCF-7, T47D, MDA-MB-231, MDA-MB-468, MDA-MB-435, SK-BR-3, and ZR-75-30) and a nonmalignant breast cell line (HBL-100) were used in this study [24]. All cell lines were maintained in RPMI 1640 (Gibco-BRL, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories, Linz, Austria), 100 U/mL penicillin, and 100 μg/mL streptomycin, and maintained at 37 °C in a humidified atmosphere containing 5% CO_2 [25]. Fresh breast tumor tissues, adjacent non-cancerous tissues, and normal breast tissues were obtained from patients treated by primary surgery at the First Affiliated Hospital Surgery Department of Chongqing Medical University (Chongqing, People's Republic of China). All samples were evaluated and histologically diagnosed by expert pathologists. Tumor grading was achieved by staining with hematoxylin and eosin (H&E). Clinical data were also obtained, including age, stage, receptor status, menstrual and tumor differentiation. For the use of these clinical materials for research purposes, prior consent of the patient and approval from the Ethics Committee of Chongqing Medical University were obtained.

2.2. Construction and transfection of the plasmid expression vectors

Based on the known Livin gene sequence in GenBank (No.NM-022161,No.NM-139317), we selected 3 interfering sequences (shRNA1: 5′-GGTG AGGTGCTTCTTCTGC-3′; shRNA2: 5′-GGCCTGGACACCTGCA GAG-3′; shRNA3: 5′-GGAAGAGACTTTGTCCACA-3′) according to the design principles of Reynolds [26]. The 19-nt oligonucleotide, 5′-GAC TTCATAAGGCGCATGC-3′,which had no significant homology to any known human mRNA in the database, was used as a negative control. Double oligonucleotide chains with complementary shRNA coding sequences were obtained from Genesil Biotechnology Company (Genesil Corp, Wuhan, China). We cloned the shRNA using pGenesil-1 vector. The recombinant vectors were named Livin-shRNA1, Livin-shRNA2, and Livin-shRNA3. Quantitative PCR (qPCR) and western blot analysis were performed to determine the Livin knockdown efficiency [27]. For the Livin overexpression studies, the Livin coding sequence was subcloned into the pcDNA3.1 plasmid according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The following primers were used for PCR amplification of the Livin coding sequence: forward, 5′-CACCATGACTTCCAAGCTGGC-3′; reverse, 5′-TTATGAATTCTCAGCCCT CTTC-3′. The plasmids were transfected into breast cancer cells using Lipofectamine 2000 transfection reagent (Invitrogen). Stably transfected cells were identified by selection for 3 weeks with 1 mg/mL G418.

2.3. Real-time reverse transcription (RT)-PCR

The following PCR methods were conducted as previously described [28]. Total RNA was extracted 48 h after transfection using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Real-time PCR samples were prepared using the SYBR premix Ex Taq kit (TaKaRa, Dalian, China) and amplification was performed on an ABI Prism 7000 detection system (Applied Biosystems, Foster City, CA, USA) according to the conditions recommended by the manufacturer. The relative expression levels of the target genes were calculated by the 2ΔCt method (the Ct of a housekeeping gene–the Ct of the target gene). The following primers were used: Livin, forward: 5′-CTGGGACCCGTGGGAAGAAC-3′, reverse: 5′-TCCTGGGCACTTTCAG ACTG-3′; β-actin, forward: 5′-TCACCCACACTGTGCCCATCTATGA-3′, reverse: 5′-GTAGCCACGCTCGGTCAGGATCTTC-3′.

2.4. Immunohistochemistry

Immunohistochemical staining for Livin was performed as described previously [20]. Immunohistochemistry was performed using a primary Livin antibody (Santa Cruz, CA, USA). Briefly, sections were deparaffinized, subjected to microwave antigen retrieval for 15 min in a sodium citrate solution (pH 6.0), and then incubated with 3% hydrogen peroxide to block endogenous peroxidase activity. The sections were incubated with the primary antibody (1:1000 dilution) overnight at 4 °C, followed by secondary antibody (Promega, Madison, WI, USA) incubation (1:2000 dilution) at 37 °C for 1 h. Finally, the slides were counterstained with hematoxylin. A negative control was included by replacing the primary antibody with PBS.

2.5. Western blot analysis

Western blot analysis was performed as described previously [28]. The antibodies used included rabbit anti-Livin (SC-30161), anti-phospho-AKT (SC-33437), anti-AKT (SC-1618), mouse anti-fibronectin (SC18827) from Santa Cruz Biotechnology, rabbit anti-vimentin (BS1776), anti-E-cadherin (BS1098), and anti-N-cadherin (BS222) from Bioworld Technology, and anti-rabbit peroxidase-conjugated secondary antibodies from Promega.

2.6. In vitro cell proliferation assay

The cells (1×10^4) were seeded onto 96-well plates. The respective recombinant plasmids were transfected into the cells and 20 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well after 1–7 days. After 4–6 h of incubation at 37 °C, 100 μL of DMSO was added and mixed well. Plates were agitated on a microtiter plate shaker to create a homogeneous solution, and the optical densities (OD) at 490 nm were determined by using an automated spectrophotometric plate reader (Bio-Tek ELX808, Winooski, VT, USA). The proliferation curve of each group was plotted based on the absorption values [26].

2.7. Colony-forming assay

Log-phase cells were seeded in triplicate onto 6-well plates with 2 mL of complete media (500 cells/well) and incubated at 37 °C in a humidified incubator. Plates were initially examined using light microscopy to confirm that only single cells without clumps had been plated. Seven to ten days after plating, the colonies were fixed with 4% formaldehyde and stained with 5% Giemsa [27].