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Sinomenine reduces growth and metastasis of breast cancer cells and improves the survival of tumor-bearing mice through suppressing the SHh pathway

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ABSTRACT

In this study, the suppressive effect of sinomenine on the activation of SHh and the progression of breast cancer metastasis in vitro and in vivo was investigated. MDA-MB-231 breast cancer cells were treated with sinomenine and/or cyclopamine a proven SHh inhibitor. Sinomenine and cyclopamine both suppressed cell proliferation and migration, but sinomenine had a stronger suppressive effect in MDA-MB-231. In addition, sinomenine could suppress the activation of NF-κB and SHh signaling pathways, but cyclopamine could not suppress the activation of NF-κB. Subsequently, a mouse breast cancer-lung metastasis model was established. Our data on tissue examination and gene detection showed that SHh signaling was markedly activated in the metastatic model mice. The progression of lung metastasis was suppressed when mice were fed sinomenine and/or cyclopamine, while sinomenine had a stronger suppressive effect than cyclopamine in the model mice. In conclusion, sinomenine has a better effect than cyclopamine on the inhibition of breast cancer metastasis to lung in vivo and vitro, and inhibits NF-κB activation and NF-κB-mediated activation of SHh signaling pathway.

1. Introduction

Breast cancer, especially metastatic breast cancer, is the greatest cancerous threat to women [1]. The five-year survival rate of the patients with metastatic breast cancer is relatively low. For instance, the five-year survival rate of bone metastasis is about 16%, that of lung metastasis is about 12%, and that of liver metastasis is little short of zero [2,3]. Therefore, it is very important to clarify the regulatory network of breast cancer metastasis and develop targeted drugs to suppress its metastasis.

The Hedgehog (Hh) signaling pathway is one of the key regulators of cell growth and differentiation during embryonic development [4]. Sufficient levels of Hh protein expression cause phosphorylation of smoothened (SMO) at the carboxyl terminal amino residues and unlocks the combination of SMO with Patched 1 receptor 1 (Ptch1) [5,6]. Then their downstream transcription factor Gli is activated and translocates into the nucleus, activating transcription of multiple genes [7]. Sonic hedgehog (SHh) is the best studied ligand of the hedgehog signaling pathway, which plays a key role in regulating vertebrate organogenesis [8]. Recently, SHh has been implicated in the development of some solid cancers, including tumors in brain, skin, esophageal, gastric, pancreatic and muscular tissues [8–12]. In breast cancer, SHh promoted glycolysis and proliferation of breast cancer cells, and drove its malignant potential [13]. Inhibition of SHh has been regarded as a potential therapeutic approach in patients with breast cancer [14]. Moreover, high expression of SHh signaling pathway genes was associated with an increased risk of recurrence of breast carcinoma [15,16].

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a vital transcription factor involved in the regulation of multiple cytobiological processes, including cell proliferation, apoptosis, adhesion and secretion [17]. Previous studies have revealed that NF-κB is required in the epithelial-mesenchymal transition (EMT) and played a key role in the invasion and metastasis of several cancers including breast cancer [18,19]. Several recent studies indicated that NF-κB crossacted with the SHh signaling pathway and dominated the activation of SHh in glioblastoma and colon cancer [20,21]. Activation of NF-κB

Abbreviations: Hh, Hedgehog; SMO, smoothened; Ptch1, Patched 1 receptor 1; SHh, sonic hedgehog; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; EMT, epithelial-mesenchymal transition; qPCR, quantitative polymerase chain reaction; IHC, hematoxylin-eosin, immunohistochemistry; Sino, sinomenine; Cyclo, cyclopamine; IL-4, interleukin 4; CUEDC2, CUE domain containing 2

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signaling induced SHh expression and played an imported role in the initiation of gastritis and invasiveness of human colon cancer cells [22]. However, it is still unclear if NF-κB is associated with SHh in breast cancer cells.

Our previous study demonstrated that sinomenine, an isoquinoline well known for its effect on anti-inflammation and apoptotic induction, suppressed breast cancer cell invasion and metastasis, acting as an indirect inhibitor of the NF-κB pathway in vitro [23]. In this study, the role of sinomenine on the progression of metastatic breast cancer in vivo was explored. A mouse model of breast cancer lung metastasis was established and the effect of sinomenine on the NF-κB-mediated activation of SHh pathway was investigated. We found that sinomenine suppressed the activation of SHh through blocking the NF-κB pathway and alleviated the progression of breast cancer lung metastasis in mice in vivo.

2. Materials and methods

2.1. Cell culture and treatment

The MDA-MB-231 human triple negative breast cancer cell lines were cultured in RPMI-1640 medium supplemented with 10% FBS (Invitrogen, Carlsbad, CA). Stock cultures were maintained in 25-cm^2 flasks (Corning Life Sciences, Lowell, MA). The cells were subcultured when they reached ~80% confluence. 0.5 mM sinomenine or 10 mM cyclopamine (both purchased from Sigma-Aldrich and dissolved in DMSO) was used to treated the cells.

This study was approved by the Ethical Committee of Xi'an Jiaotong University. All animal experiment protocols were approved by the Institutional Animal Care and Use Committee of the Xi'an Jiaotong University. After experimentation, to minimize pain without drugs, the mice were rapidly euthanized by cervical dislocation and decapitation by an experienced animal handler.

2.2. Cell viability detection

Cell proliferation was evaluated using the MTT (Sigma, St. Louis, MO) assay. After treatment, the cells were incubated for 48 h at 37 °C before adding the MTT reagent to each well at a final concentration of 0.5 mg/mL and incubated at 37 °C for 4 h. After medium removal, 500 μL of dimethyl sulfoxide was added to each well. Viable cells were measured by absorbance at a 550 nm wavelength using a microplate reader (BioRad, Hercules, CA).

2.3. In vitro migration assay

A wound-healing assay was conducted to assess cell migration. The cells were grown in 24-well plates (3.5×10^4/well) and an artificial wound was created 24 h after treatment using a 10-μL pipette tip. To visualize cell migration and wound healing, images were taken at 0 h and 24 h respectively. Images were captured at 40× magnification under an Olympus IX70 light microscope (Olympus Corporation; Osaka, Japan) and the wound area of each group were measured and calculated.

2.4. Quantitative polymerase chain reaction (qPCR)

Total RNA of each clone was extracted using TRIzol (Invitrogen); reverse transcription and cDNA amplification were performed according to the manufacturer's instructions in the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara Biotechnology, Dalian, China). The qPCR primers used in this study were designed and synthesized by Genscript Inc. (Nanjing, China) (Table 1). Each individual sample was run in triplicate wells. All mRNA amounts were normalized to 18S RNA. Data were analyzed using the comparative $2^{-\Delta\Delta Ct}$ method.

Table 1
The primers of qRT-PCR.

Gene names	Primer sequences
Shh	Sense primer:5′- GTGGCCGAGAAGACCCTA-3′ Anti-sense primer: 5′- CAAAGCGTTCAACTTGTCCTTA-3′
SMO	Sense primer: 5′- TCGCTACCCTGCTGTTATTC -3′ Anti-sense primer: 5′- GACGCAGGACAGAGTCTCAT -3′
Ptch1	Sense primer: 5′-TGACCTAGTCAGGCTGGAAG-3′ Anti-sense primer: 5′-GAAGGAGATTATCCCCCTGA-3′
Gli	Sense primer: 5′-CTGGATCGGATAGGTGGTCT-3′ Anti-sense primer: 5′-CAGAGGTTGGGAGGTAAGGA-3′
GAPDH	Sense primer: 5′-CGTCTTCACCACCATGGAGA-3′ Anti-sense primer: 5′-CGGCCATCACGCCACAGTTT-3′

2.5. Western blot analysis

A total of 50 μg of protein from each sample was separated by 12% SDS-PAGE. Afterwards, proteins were transferred to PVDF membranes (Millipore, Boston, MA) at 250 mA for 2 h. After transfer, the membranes were blocked using 5% non-fat dried milk in Tris-buffered saline with Tween-20 (TBS-T). The membranes were then incubated with primary antibodies (Abcam; Cambridge, UK): anti-IκB (1:400 dilution), anti-p-IκB (Ser32/36; 1:500), anti-IKKβ (1:500), anti-p-IKKβ (Ser 181; 1:300), anti-SHh (1:500), anti-Ptch (1:300), anti-SMO (1:500), anti-Gli (1:500), anti-MMP-2 (1:500), anti-vimentin (1:300). For the loading control, the membranes were probed with anti-β-actin (1:800) or anti-GAPDH antibodies (1:600) in TBS-T for 1 h. The membranes were then incubated with HRP-conjugated anti-rabbit secondary antibody (1:2000) in TBS-T for 1 h. Finally, the membranes were incubated with ECL chemical luminescence solution (Boehringer Mannheim; Mannheim, Germany), then exposed in a ChemiDoc XRS imaging system and analyzed with Quantity One software (BioRad).

2.6. NF-κB nuclear translocation

NF-κB location in the cell was detected using an Olympus FV-1000 laser scanning confocal microscope (Olympus Corporation; Osaka, Japan). NF-κB was detected in the green channel and the nuclei were stained with the fluorescent dye DAPI and detected in the blue channel.

2.7. Hematoxylin-eosin (HE) staining and immunohistochemistry (IHC) assay

The lung tissues were collected from each mouse. Morphological changes in the lung were detected by H&E staining as described elsewhere. Streptavidin biotin labeled IHC assays were used to detect the expression of SHh protein. The tissue samples were paraffin embedded and sectioned to a thickness of 10 μM. The sections were dewaxed and treated with 0.3% H_2O_2 in methanol for 10–20 min. After washing with wash buffer, the slices were antigen repaired with vacuum negative pressure. The slices were treated in the following order (note that after each incubation, the slices were washed three times with PBS): serum incubation for 10 min, SHh primary antibody incubation for 45 min, secondary antibody (goat anti-mouse IgG) incubation for 30 min, streptavidin biotin-H_2O_2 incubation for 10 min, and Harris dye staining for 10 min. Finally, the slices were dehydrated, cleared and sealed. The slices were observed with an XSP-4C binocular microscope (Bingyu Optical Instrument CO., LTD.; Shanghai, China).

2.8. Preparation of breast cancer-lung metastasis mouse model and drugs feeding

One hundred twenty BALB/C male nude mice (aged about 4 months, weighed about 35 g) were used in this experiment. A total of