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Sonic hedgehog stimulates glycolysis and proliferation of breast cancer cells: Modulation of PFKFB3 activation

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ABSTRACT

Sonic hesgehog (Shh) signaling has been reported to play an essential role in cancer progression. The mechanism of Shh involved in breast cancer carcinogenesis remains unclear. The present study sought to explore whether Shh signaling could regulate the glycolytic metabolism in breast cancers. Overexpression of the smoothed (Smo) and Gli-1 was found in human primary breast cancers. The expressions of Shh and Gli-1 correlated significantly with tumor size and tumor stage. In vitro, human recombinant Shh (rShh) triggered Smo and Gli-1 expression, promoted glucose utilization and lactate production, and accelerated cell proliferation in MCF-7 and MDA-MB-231 cells. Notably, rShh did not alter 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) expression but augmented PFKFB3 phosphorylation on ser^{461}, along with elevated fructose-2,6-bisphosphate (F2,6BP) generation by MCF-7 and MDA-MB-231 cells. This effect could be dampened by Smo siRNA but not by Gli-1 siRNA. In addition, our data showed the upregulated expressions of MAPK by rShh and elevatory PFKFB3 phosphorylation by p38/MAPK activated kinase (MK2). In conclusion, our study characterized a novel role of Shh in promoting glycolysis and proliferation of breast cancer cells via PFKFB3 phosphorylation, which was mediated by Smo and p38/MK2.

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1. Introduction

The Hedgehog (Hh) family secreted proteins includes Sonic (Shh), Indian (Ihh), and Desert (Dhh) [1], a group of proteins firstly found to be involved in animal development [2]. Hh pathway activation is initiated by the binding of these Hh ligands. Then, the cell surface receptor Ptch family (Ptch1, Ptch2) and the smoothened (Smo) transmit the signal into the cell, following with the signal transduction into the nucleus by the Gli-family factors (Gli-1, Gli-2) [3–5]. Deregulation of the Shh signaling pathway has been shown to affect developmental process, tissue regeneration, stem cell renewal and cancer pathology [6]. In human hepatocellular carcinoma (HCC), Shh signaling pathway mediates cancer cells invasion and metastasis by increasing MMP-9 expression via ERK pathway [7]. Moreover, overexpression of Shh was found in breast cancer

tissues and survival analysis suggested that Shh overexpression was a poor prognosis indicator for breast cancers [8]. These evidence characterized that Shh overexpression was a critical event in breast carcinogenesis [8,9]. In addition, Shh activation induced transcription of hexokinase 2 (HK2) and pyruvate kinase M2 (PKM2) and caused a robust increase of glycolytic metabolism in medulloblastoma [10]. The process is mediated by the canonical activation of the Gli transcription factors [10]. Nevertheless, the exact role of Shh signaling in breast cancer remains unclear.

The Warburg effect is a description of metabolism in malignant tumors. Cancer cells are inclined to produce energy by glycolysis rather than by oxidative phosphorylation (OXPHOS) [11]. The Warburg phenotype had stronger associations with triple negative breast carcinomas (TNBC) than luminal breast cancers, suggesting a correlation between metabolic phenotype and the biology of breast cancer [12]. Furthermore, the metabolic phenotype of breast cancer stem cells (BCSCs) and their differentiated progeny was also investigated [13]. BCSCs consume more glucose, produce less lactate, and have higher ATP content compared to their differentiated progeny [13].

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Fructose-2,6-bisphosphate (F2,6BP), an allosteric activator of Phosphofructokinase-1 (PFK-1), plays a key regulation role in glycolytic flux [14,15]. Steady state levels of F2,6BP are maintained by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB) family enzymes, especially by PFKFB3 [16]. Some anti-oncogenes may modulate PFKFB3 expression. In the embryonic fibroblasts, phosphatase and tensin homolog (PTEN) knock-out was shown to significantly raised the protein levels of PFKFB3 and concentrations of F2,6BP [17]. PFKFB3 expression has been demonstrated to contribute to tumor growth and metastasis. Heterozygous deletion of the PFKFB3 gene has been found to reduce both the glucose metabolism and growth of Ras-transformed tumors in syngeneic mice [18].

In this study, we sought to explore whether Shh signaling could regulate the glycolytic metabolism in breast cancers. We found that Shh and Gli-1 were overexpressed in breast carcinomas and their expressions were correlated with critically clinicopathological factors. In vitro, Shh stimulated glycolysis and proliferation of breast cancer cells via PFKFB3 activation, which was mediated by Smo and p38 MAPK/MK2.

2. Materials and methods

2.1. Collection of human samples

Samples of breast tumor tissues and normal breast tissues (n = 117) were obtained from the patients (mean age 52.4 years, range 27–59 years) enrolled in the First Affiliated Hospital of Zhengzhou University between 2011 and 2014. Human samples were prepared and quickly frozen in liquid nitrogen. The study was reviewed and approved by the Ethical Committee of the First Affiliated Hospital of Zhengzhou University and carried out in according with the declaration of Helsinki. Written informed consent was obtained from each patient.

2.2. Cell culture and treatment

Breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Invitrogen) under a 37 °C, 5%CO_2 circumstance.

For human recombinant Shh (rShh; Novoprotein, Summit, NJ, USA) treatment, 1×10^4/well breast cancer cells were treated with 0, 5, 10 μg/mL rShh for 1 h and then prepared for further analysis. To inhibit the activity of MAPK in MCF-7 and MDA-MB-231 cell lines, a list of specific inhibitors including SB203580 (10 nM), PF3644022 (10 nM), UO126 (10 nM), LY294002 (10 nM) were added into the medium and incubated for 30 min.

2.3. Immunohistochemistry staining

Tumor tissues and normal control samples were detected with anti-Smo (1:400; Santa Cruz Biotech, Santa Cruz, CA, USA) and anti-Gli-1 (1:300; Santa Cruz Biotech) polyclonal antibody. Image acquisition was performed with a microscope (Olympus, Tokyo, Japan) and assessed by a pathologist. Immunostaining intensity (1, weak; 2, moderate; 3, intense) and the ratio of positive cells (0, <5%; 1, 5–25%; 2, 26–50%; 3, 51–75%; 4, >75%.) were calculated in at least five fields (400×). The scores of each sample were multiplied to give a final score of 0, 1, 2, 3, 4, 6, 8, 9, or 12.

2.4. siRNA transfection

For Smo expression and Gli-1 expression silencing, small interfering RNAs (siRNAs) for control (non-specific siRNA) and Smo or Gli-1 siRNA were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA), MCF-7 and MDA-MB-231 cells were transfected with control, Smo or Gli-1 siRNA using Lipofectamine 2000(Invitrogen) according to the manufacturer's instructions. At 48 h after transfection, cells were harvested for further analysis.

2.5. RNA extraction and qRT-PCR

Total RNA was extracted from tissues and breast cancer cells using Trizol reagent (Takara, Dalian, China). For PFKFB3 expression analysis, the cDNA was generated using HiScript 1st Strand cDNA Synthesis Kit (Vazyme; Nanjing, China). Expression quantification of the target genes (Smo, Gli-1 and PFKFB3) was carried out on a 7500 Fast Dx Real-Time PCR System (Applied Biosystems) with HiScript® Q RT SuperMix for qPCR(Vazyme; Nanjing, China). The primers including forward: 5′-CTG TA AGA GGA CGT GCA GA-3′, reverse: 5′-AGG GTG AAG AGC GTG CAC TG-3′ (for Smo); forward: 5′-TCA CTA CTA GAG TCC CAA CC-3′, reverse: 5′-GCA GAT GTG AAG CGA TAT GA-3′ (for Gli-1); forward: 5′-AGA ACT TCC ACT CTC CCA CCC AAA-3′, reverse: 5′-AGG GTA GTG CCC ATT GTT GAA GGA-3′ (for PFKFB3) and forward: 5′-GAG CTA CGA GCT GCC TGA CG-3′, reverse: 5′-CCT AGA AGC ATT TGC GGT GG-3′ (for β-actin) were used. All experiments were repeated for three times.

2.6. Western blot

Total proteins were extracted and separated by SDS-PAGE, and western blot analysis was performed according to standard procedures. β-actin was used as the reference gene for protein quantification. Monoclonal antibody for Smo (1:1000, Santa Cruz Biotech), Gli-1(1:1000, Santa Cruz Biotech), PFKFB3 (1: 1500, Sigma–Aldrich, St. Louis, MO, USA), p-p38 MAPK (1:1000, Sigma–Aldrich), p-MK2 (1:1500, Santa Cruz Biotech), p-p42/p44 MAPK (1:1000, Santa Cruz Biotech), p-ERK1/2 (1:1500, Santa Cruz Biotech), p-Akt (1:1500, Santa Cruz Biotech) and β-actin (1:2000, Abcam, Cambridge, UK) were employed for the immunodetection. Polyclonal antibodies including anti-p-PFKFB3 (ser478) and anti-p-PFKFB3 (ser461) were obtained according to a previous report [19]. Goat anti-mouse IgG (1:10 000) was used as the second antibody following with enhanced chemiluminescence (ECL, Amersham Pharmacia, NJ, USA) detection.

2.7. Glucose utilization assay

Glucose Assay Kit (Abcam) was employed to assay the concentration of D-glucose in medium of MCF-7 and MDA-MB-231. Glucose utilization by the cells was calculated as the difference between the starting glucose concentration and the final glucose concentration in medium. All experiments were repeated for three times.

2.8. Lactate and F2,6BP measurements

Lactate levels were measured using a lactate oxidase-based colorimetric assay read at 540 nm according to the manufacturer's instructions (Beyotime, Wuxi, China) and normalized to cell numbers. Before the measurement of total intracellular F2,6BP, 1×10^4 cells were trypsinized and washed twice with PBS. The F2,6BP concentration was normalized to total cellular protein as measured by the bicinchoninic acid (BCA) assay. All experiments were repeated for three times.

2.9. Cell proliferation assays

To evaluate cell proliferation, 1×10^4/well breast cancer cells were treated with 0, 5, 10 μg/mL rShh for 1 h before plating into