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Evodiamine inhibits STAT3 signaling by inducing phosphatase shatterproof 1 in hepatocellular carcinoma cells

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ABSTRACT

The activation of signal transducer and activator of transcription signaling 3 (STAT3) has been linked with the survival, proliferation, angiogenesis and immunosuppression of hepatocellular carcinoma cells (HCCs). Agents that can suppress STAT3 activation have potential to be cancer therapeutics. In this study, we investigated the inhibitory effect of evodiamine on STAT3 pathway *in vitro* and the anti-tumor effect of evodiamine in vivo in HCC. We found that evodiamine suppressed both constitutive and interleukin-6 (IL-6)-induced activation of STAT3 tyrosine 705 (Tyr^{705}) effectively. The phosphorylation of Janus-activated kinase 2 (JAK2), Src and extracellular regulated protein kinases 1/2 (ERK1/2) were also suppressed by evodiamine. Interestingly, treatment of cells with sodium pervanadate abrogated the inhibition of evodiamine on IL-6-induced STAT3 (Tyr^{705}) activation indicating the involvement of protein tyrosine phosphatases. Indeed, further studies demonstrated that evodiamine induced the expression of phosphatase shatterproof 1 (SHP-1). Moreover, inhibition of SHP-1 gene by small interference RNA abolished the ability of evodiamine to inhibit IL-6-induced STAT3 (Tyr^{705}) activation. Evodiamine also suppressed STAT3 DNA binding activity and down-regulated the expression of STAT3-mediated genes leading to the suppression of proliferation, induction of cell apoptosis and cell cycle arrest. In vivo, evodiamine significantly inhibited tumor growth in a subcutaneous xenograft model with HepG2 cells. In summary, evodiamine blocked STAT3 signaling pathway by inducing SHP-1 and exhibited anticancer effect *in vitro* and in vivo.

1. Introduction

The STAT family of transcription factors transduce signals from a variety of extracellular stimuli and mediate inflammation, cell survival, differentiation, and proliferation [1,2]. Among STAT family members, STAT3 is the most frequently associated with cell growth disorder and neoplasia [3]. Hepatocellular carcinoma (HCC) is the fifth common cancer and the third leading cause of cancer-related deaths worldwide [4]. STAT3 was found to be activated in the majority of HCCs with poor prognosis but not in surrounding non-tumor tissue or in normal liver [5]. Phosphorylation of Tyr^{705} of STAT3 plays a central role in activating downstream genes [6]. Briefly, STAT3 phosphorylation on Tyr^{705} is mediated by Janus-activated kinases (JAKs) and Src family kinases [7], resulting in homodimerization or heterodimerization of STAT3, enabling nuclear localization and DNA binding [8], and regulating downstream genes involved in controlling cell cycle progression and programmed cell death (e.g., cyclin D1, Mcl-1, Bcl-2, survivin and XIAP), and in the regulation of angiogenesis (e.g., VEGF [9], HIF1-α and MMP-9). IL-6 is a pleiotropic cytokine that can activate STAT3 signaling pathway [10]. Malaguarnera et al. [11] demonstrated a significant increase in serum IL-6 concentrations in HCC patients compared with controls, as well as an elevated positive correlation between IL-6 and the size of the tumor. Corazza et al. proposed that IL-6 could be considered as a promising tumor marker for HCC [12]. The inhibition of aberrant STAT3 activation by genetic or pharmacological approaches has repeatedly been demonstrated to result in growth inhibition, apoptosis *in vitro* [13], as well as tumor growth and metastasis inhibition in vivo [14,15] in HCC. Therefore, agents that can suppress STAT3 pathway may have a potential to prevent and treat HCC which harbor excessive STAT3 activation.

Evodiamine, an alkaloid isolated from the dried, unripe fruit of *Evodia rutaecarpa* (*Juss.*) *Benth*, has been reported to have immunoregulatory effect in mice, anti-angiogenesis effect [16], anti-inflammatory effect [17], inhibitory effect on adipocyte differentiation [18] and adipogenesis. Evodiamine also exhibited anti-tumor

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activity in a number of human cancers [19–21]. However, effects of evodiamine on STAT3 signaling pathway have not been reported. We have analyzed the effects of evodiamine on the JAK2/STAT3 signaling pathway in hepatocellular carcinoma cells. We found that evodiamine blocked STAT3 cascade and this blockage was mediated by inducing the expression of a protein tyrosine phosphatase SHP-1. Evodiamine also induced HCC apoptosis and inhibited tumor growth in a subcutaneous xenograft model with HepG2 cells in vivo. Evodiamine may be a candidate for HCC therapy through blocking constitutive and IL-6-induced STAT3 signaling.

2. Materials and methods

2.1. Cell lines and cultures

Human hepatocellular carcinoma cell lines HepG2, Bel-7402, QGY-7701 and normal hepatocellular cell line LO-2 were obtained from the Cell Bank of the Shanghai Institute of Biochemistry & Cell Biology were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). All cells were cultured in a humidified atmosphere with a 5% CO_2 incubator at 37 °C.

2.2. Agents and antibodies

Evodiamine was obtained from the national institute for the control of pharmaceutical and biological products (Beijing, China). A 10 mM solution of evodiamine was prepared in dimethyl sulfoxide (DMSO), stored as small aliquots at 4 °C. STAT3, phospho-STAT3 (Tyr^{705}), phospho-JAK2, Src, phospho-Src, SHP-1, SHP-2, Bcl-2, Mcl-1, cyclin D1, VEGF, survivin, XIAP, HIF1-α, MMP-9, ERK1/2, phospho-ERK1/2, AKT, phospho-AKT, caspase-3, caspase-8, PARP, GAPDH antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase-conjugated secondary antibodies and NE-PER® Nuclear and Cytoplasmic Extraction and LightShift Chemiluminescent EMSA kit were obtained from Santa Cruz Biotechnology (CA, USA). Maxima® SYBR Green/ROX qPCR Master Mix (2×) and Maxima® First Strand cDNA Synthesis Kit were purchased from Fermentas life science (Fermentas, MBI). MTT (methyl thiazolyl tetrazolium), RNase (ribonuclease), sodium-othrovanadate, propidium iodide (PI) and recombinant human IL-6 were purchased from Sigma-Aldrich (St. Louis, MO, USA). AG490 was obtained from Tocris Bioscience (Bristol, UK).

2.3. Immunoblot assay

Culture cells in six-well plates at a density of 5×10^6 cells/mL in a CO_2 incubator overnight and treated with evodiamine. The total protein were prepared as previously described [22]. The equalized amounts of proteins from each sample were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in 1% (w/v) albumin from bovine serum (BSA) for 2 h, and then incubated with primary antibody overnight at 4 °C. Then the membranes were incubated with secondary antibody conjugated with IgG horseradish peroxidase (HRP) for 1 h at room temperature and immune complexes were detected by the enhanced chemiluminescence system. GAPDH served as a loading control.

2.4. Cytotoxicity assay

HepG2 cells and LO-2 cells were incubated in triplicate in a 96-well plate at a density of 1×10^4 cells with 100 μL culture medium per well in the presence or absence of indicated concentrations of evodiamine for 24 h, 48 h, and 72 h. After which, cell viability was determined by the MTT dye uptake method as described earlier [23].

2.5. Electrophoretic mobility shift assay (EMSA)

STAT3-DNA binding activity was analyzed by EMSA using a biotin-labeled, double-stranded STAT3 consensus-binding motif probe (5′-GATCCTTCTGGGCCG TCCTAGATC-3′ and 3′-CTAGGAAGACCCGGCAGGATCTAG-5′). Nuclear protein extracts were prepared from evodiamine treated HepG2 cells and incubated with biotin-labeled probe. STAT3-DNA complex formed was separated from free oligonucleotide on 5% nondenaturing polyacrylamide gels, then transferred to nylon membranes, and cross-linked for 15 min under a hand-held UV lamp. Cross-linked, biotin-labeled DNA was detected using the Chemiluminescent Nucleic Acid Detection Module (Pierce Biotechnology, inc.).

2.6. Reverse-transcription polymerase chain reaction

HepG2 cells were treated with different concentrations of evodiamine for 5 h, then treated cells were washed with PBS, total RNA was extracted from the treated cells using trizol reagent (Invitrogen, Carlsbad, CA) and then RNA was converted to cDNA by reverse transcriptase (Transgene, China) according to the manufacturer's instruction. The relative expression of SHP-1 was analyzed using reverse-transcription polymerase chain reaction (RT-PCR) with GAPDH as an internal control. The RT-PCR reaction was processed according to previous described [24]. PCR products were run on 4% agarose gel and then stained with ethidium bromide. Stained bands were visualized under UV light and photographed.

2.7. SHP-1 silencing

HepG2 cells were plated in six-well plates for 24 h prior to transfection so that the monolayer cell density reaches to the optimal ~50% confluency before transfection. siRNA for SHP-1 transfection were performed by Lipfectamine 2000 (Invitrogen). After transfection for 6 h, transfection medium was replaced with growth medium and incubated for 48 h, cells were treated with evodiamine for 5 h and whole-cell extracts were prepared to examine the protein levels of SHP-1, phospho-STAT3 and STAT3 by Western blot analysis.

2.8. Cell cycle analysis

HepG2 cells were first synchronized by serum starvation and then exposed to different concentrations of evodiamine for 24 h. Thereafter, treated cells were fixed and incubated with RNAse and propidium iodide (PI) in PBS. Cell cycle distribution was analyzed with a FAC-Scan laser flow cytometer (FAC-SCalibur, Becton Dickinson, USA), The data were analyzed using the software CELL Quest.

2.9. Real-time qPCR analysis

Total RNA was extracted from the xenograft tumors using trizol reagent (Invitrogen, Carlsbad, CA) and then RNA was converted to cDNA by reverse transcriptase according to the manufacturer's instruction. Real-time qPCR analysis for SHP-1, Mcl-1, COX-2 and VEGF mRNA expression was performed using SYBR Green probes and an ABI 7500. SHP-1, Mcl-1, COX-2 and VEGF mRNA expression were normalized against GAPDH expression.

2.10. Immunohistochemical (IHC) analysis

Immunohistology analysis was carried out using paraffin section. Paraffin section were incubated in a blocking solution (10% donkey serum +5% nonfat dry milk +4% BSA +0.1% Triton X-100) for 10 min and then hydrated sections were incubated at 4 °C overnight with anti-phosphor-STAT3 (Tyr^{705}) and anti-STAT3 antibody respectively. After washing with PBS, the sections were incubated with diluted (1:200) biotinylated secondary antibody for 30 min. Subsequently, the slides were washed again in PBS and incubated for 30 min with the preformed avidin-horseradish peroxidase macromolecular complex. Development of peroxidase reaction was achieved by incubation in 0.01% 3, 3-diaminobenzidine tetrahydrochloride (DAB) in PBS containing 0.01% hydrogen peroxide for approximately 5 min at room temperature. Sections were then washed thoroughly in tap water, counterstained in haematoxylin, dehydrated in absolute alcohol, cleared in xylene and mounted in synthetic resin for microscopic examination.

2.11. Animal experiments

All animal experiments were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. Cultured HepG2 cells (5×10^6) suspended in 0.2 mL PBS were injected into the right flank of 5-week-old female BALB/c athymic nude mice. The mice were kept in a pathogen-free environment, 5–7 days later when the tumor volume reached 100 mm^3, the mice were divided into four groups (control group, 5-FU group, low dose evodiamine group and high dose evodiamine group) in a manner to equalize the mean tumor among the four groups ($n = 7$ each). Mice of 5-FU group were given as an intraperitoneal injection fifth a week and the injection volume was 0.1 mL. Evodiamine at 20 mg/kg and 10 mg/kg dose suspended in 0.5% carboxymethyl cellulose (CMC) was given as gavage to mice daily for 4 weeks, mice of control group were given 0.1 mL 0.5% CMC solution. The tumor size was measured in two orthogonal directions using calipers every three days, and the tumor volume (mm^3) was estimated using the equation length × $(\text{width})^2 \times 0.5$. Four weeks later, the mice were sacrificed and the tumors were resected.

2.12. Statistical analysis

Statistical difference was analyzed by two-way Student's t-test. $P < 0.05$ was considered statistically significant. The values are expressed as the mean ± SD. Three or more separates experiments were performed.