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Research article

Shikonin regulates C-MYC and GLUT1 expression through the MST1-YAP1-TEAD1 axis

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ABSTRACT

The general mechanism underlying the tumor suppressor activity of the Hippo signaling pathway remains unclear. In this study, we explore the molecular mechanisms connecting the Hippo signaling pathway with glucose metabolism. We have found that two key regulators of glycolysis, C-MYC and GLUT1, are targets of the Hippo signaling pathway in human leukemia cells.

Our results revealed that activation of MST1 by the natural compound shikonin inhibited the expression of GLUT1 and C-MYC. Furthermore, RNAi experiments confirmed the regulation of GLUT1 and C-MYC expression via the MST1-YAP1-TEAD1 axis. Surprisingly, YAP1 was found to positively regulate *C-MYC* mRNA levels in complex with TEAD1, while it negatively regulates C-MYC levels in cooperation with MST1. Hence, YAP1 serves as a rheostat for C-MYC, which is regulated by MST1. In addition, depletion of MST1 stimulates lactate production, whereas the specific depletion of TEAD1 has an opposite effect. The inhibition of lactate production and cellular proliferation induced by shikonin also depends on the Hippo pathway activity. Finally, a bioinformatic analysis revealed conserved TEAD-binding motifs in the *C-MYC* and *GLUT1* promoters providing another molecular data supporting our observations.

In summary, regulation of glucose metabolism could serve as a new tumor suppressor mechanism orchestrated by the Hippo signaling pathway.

1. Introduction

The Hippo signaling pathway is an evolutionarily conserved regulator of organ size during development and a potent tumor suppressor in adults [1–4]. To date, the molecular mechanism responsible for its anti-proliferative and pro-apoptotic activities remains poorly understood. In mammals, the Hippo pathway comprises a central kinase cassette (CKCA) and consists of the MST1/2 serine/threonine kinase, LATS1/2 protein kinase, and two scaffold proteins, SAV1 and MOB1A/1B. CKCA activity inhibits the formation of a complex between the TEAD transcription factors and the YAP/TAZ effectors in a LATS1/2-dependent manner [5,6]. YAP/TAZ activity is repressed by LATS-dependent phosphorylation, which promotes the association of YAP/TAZ with the 14-3-3 protein and subsequently sequesters the complex in the cytosol [7]. Several reports demonstrated ubiquitin-mediated proteolysis of YAP/TAZ as a consequence of this phosphorylation [3,8].

In mammals, CKCA is regulated by several upstream components. While the KIBRA-WILLIN-NF2 complex activates CKCA by an unknown mechanism, the apicobasal polarity proteins (such as SCRIB) activate CKCA and G-protein-coupled receptors, bypassing the MST1 and MST2 interaction with LATS1 and LATS2. Several other proteins (for instance AMOT, ZO, and PTPN14) directly regulate the activities of the YAP/TAZ Hippo signaling effectors by sequestration of the complex to the plasma membrane [9–11]. Actin filaments can regulate Hippo signaling in cancer cells in a Rho-dependent manner [12]. Furthermore, activation of the Hippo pathway by protein kinase A was recently discovered [13]. Therefore, the regulation of the Hippo signaling pathway represents a promising way to effective cancer treatment [14].

The oncoprotein C-MYC acts as a key developmental transcription factor and is strongly involved in the regulation of cellular metabolism and growth [15]. C-MYC is also an important factor responsible for the metabolic reprogramming of cancer cells known as the Warburg effect [16,17], which primarily depends on the activity of downstream glycolytic components including pyruvate kinase M2 (PKM2), lactate dehydrogenase A (LDHA), pyruvate dehydrogenase kinase (PDK), and monocarboxylate transporter (MCT1). Activity of these downstream glycolytic components blocks the transport of pyruvate into mitochon-

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dria and drives the conversion of pyruvate to lactate. This process supports the synthesis of important biomolecules, while simultaneously decreasing the oxidative stress resulting from mitochondrial metabolism. Targeting the activities of C-MYC at various cellular levels has proven useful during the treatments of diverse types of cancer [18,19].

Shikonin represents naphtoquinonic compound isolated from the Chinese plant *Lithospermum erythrorhizon* [20]. Shikonin treatment inhibits AKT and ERK signaling pathways and induces apoptosis through FOXO transcription factors and mitochondrial pathway [21,22]. Recent results suggest inhibition of leukemia cells proliferation induced by the shikonin analogue Quambalarine B and this inhibition depends on regulation of the C-MYC protein [23].

The present study demonstrates molecular mechanism responsible for the shikonin-driven inhibition of the C-MYC levels in leukemia cells. We have also probed effect of this mechanism on the GLUT1 expression, cellular proliferation and inhibition of lactate production triggered by shikonin treatment in leukemia cells.

2. Materials and methods

2.1. Cell lines, culture conditions, and reagents

The T-cell lymphoma-derived Jurkat cell line, clone E6.1, was obtained from the ATCC collection (ATCC, Manassas, VA, USA) and cultured in the RPMI1640 medium with L-glutamine (Lonza Group, Ltd., Basel, Switzerland) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (PAA Laboratories, Pasching, Austria) at 37 °C and 5% CO_2.

2.2. Shikonin treatment

Cells were seeded at a density of 2.5×10^5 cells/mL in 15 mL of the RPMI1640 medium and cultured overnight in 75-cm^2 cell culture flasks under standard cultivation conditions. Cells were treated with 2 μmol/L shikonin (Sigma-Aldrich) dissolved in DMSO; an equal volume of DMSO was added to control cells. Cells were harvested at indicated times, washed with PBS, and processed for analysis. For each time point, controls and treated cells were grown in individual 75-cm^2 flasks. For the assessment of GLUT1 levels, cells were incubated for 24 h with 0.5 μmol/L shikonin.

2.3. RNA isolation, reverse transcription, and RT-qPCR analysis

Cultured cells were harvested (400×*g*, 4 min), washed with PBS buffer, and lysed in a cell lysis buffer (Aurum RNA Isolation Kit, Bio-Rad Laboratories, Hercules, CA, USA). The extracted cell lysate was processed according to the manufacturer's protocol, including an in-column DNase treatment. The purified RNA was quantified and tested for the presence of contaminants with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription of 700 ng of each purified RNA sample was performed using the M-MulV *Taq* RT-PCR Kit (New England Biolabs, Ipswich, MA, USA). For individual RT-qPCR reaction mixtures, 2 μL of cDNA from the reverse transcription protocol were used. Individual reactions were run in triplicate using an iQ5 Real-Time PCR System (Bio-Rad) in final volumes of 25 μL. Specific primers and the SsoFast™ EvaGreen Supermix (Bio-Rad) were used for amplification and fluorescent detection of PCR products, respectively. The relative quantities of cDNA from treated and control cells were calculated by the Livak and Schmittgen $2^{-\Delta\Delta C_T}$ method [24]. The effects of MST1-specific siRNAs transfections and shikonin treatments on the relative quantities of *C-MYC* and *GLUT1* mRNAs are shown as the mean ± standard deviation (SD) of the percentage values of non-targeted, non-treated control cells. The human ribosomal protein *RPLP0* mRNA was used as a reference in all RT-qPCR experiments. The following primers were used in RT-qPCR for the quantification of individual mRNAs (Table 1).

Table 1
Sequences of primers used in RT-qPCR experiments.

mRNA Target	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
C-MYC	TCGGATTCTCTGCTCTCCTC	TCGGTTGTTGCTGATCTGTC
GLUT1	TCGTCGTCGGCATCCTCATC	CGGTTGATGAGCAGGAAGCG
TEAD1	CCATGTCCTCAGCCCAGATC	TTGTGAGGATCCTGGCTGCC
TEAD2	GGTGGCTTCTACGGAGTGAG	AATCTGCCGTCCTCCAGCTG
TEAD4	AAGACCCGCACCAGGAAGCA	CCTTGTCCTTAGCTGCCTGG
RPLP0	TCGACAATGGCAGCATCTAC	ATCCGTCTCCACAGACAAGG

2.4. SDS electrophoresis, immunoblotting, and cell fractionation

For whole cell lysate analyses, cells were washed with PBS supplemented with a Phosphatase Inhibitor Cocktail (Active Motif, La Hulpe, Belgium), lysed using the RIPA buffer (1% NP-40, 150 mM NaCl, 0.5% NaDOC, 0.1% SDS and 50 mM Tris-HCl pH 8) supplemented with Complete Protease Inhibitor Cocktail Tablets (F. Hoffmann-La Roche Ltd., Basel, Switzerland), and incubated for 30 min on ice. The cell lysate was cleared via centrifugation (14,000×*g*, 10 min, 4 °C). Bicinchoninic acid (BCA) assays were used to determine total protein concentration in the cell lysates (BCA Protein Assay Kit, Thermo Fisher Scientific). Nuclear and cytosolic fractions were isolated from Jurkat cells using Nuclear Extract Kits (Active Motif). SDS PAGE gels were loaded with 40 μg of protein per lane from individual samples. A Protean III apparatus (Bio-Rad) with a constant voltage of 100 V was used to run the SDS PAGE protein samples. Separated proteins were blotted onto a nitrocellulose membrane (Pall Corporation, Port Washington, NY, USA) using a Trans-Blot™ SD Semi-Dry apparatus (Bio-Rad). Protein blots were blocked for 1 h in TBS supplemented with 5% milk (Bio-Rad) and 0.05% Tween-20 (Sigma-Aldrich). Membranes were then washed with TBS containing 0.05% Tween-20 and incubated with the respective primary and secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), according to manufacturer's protocols. The following antibodies were used for immunostaining: anti-MST1 (Cell Signaling, Danvers, MA, USA), anti-phospho-(T183)-MST1 (Cell Signaling), anti-YAP1 (Sigma-Aldrich), anti-C-MYC (Cell Signaling), anti-GLUT1 (Abcam, Cambridge, UK), anti-Histone3 (Cell Signaling), anti-phospho-(S127)-YAP1 (Abcam), anti-Actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-TEAD1 (Cell Signaling).

2.5. Nuclear complex co-immunoprecipitation

Nuclear complex co-immunoprecipitation (co-IP) was performed using the Nuclear Complex Co-IP Kit (Active Motif) according to manufacturer's instructions. Briefly, the nuclei isolated from controls and cells treated for 4 h with shikonin were subjected to enzymatic shearing (room temperature, 30 min). The protein content from the nuclear fraction was quantified using BCA assays, and 300 μg of each protein sample were subjected to precipitation in a low stringency buffer with specific antibodies at 4 °C overnight on a rotator. Subsequently, suspensions of washed protein G agarose beads (50 μL, Santa Cruz Biotechnology) were added to individual precipitation mixtures and incubated for an additional 1 h on a rotator. The beads were finally washed with a reducing SDS buffer, boiled, centrifuged briefly, and subjected to SDS PAGE, followed by immunoblotting. YAP1 was detected in individual samples using the anti-YAP1 antibody.