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Yap promotes hepatocellular carcinoma metastasis and mobilization via governing cofilin/F-actin/lamellipodium axis by regulation of JNK/Bnip3/SERCA/CaMKII pathways

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ABSTRACT

Despite the increasingly important role of Hippo-Yap in hepatocellular carcinoma (HCC) development and progression, little insight is available at the time regarding the specifics interaction of Yap and cancer cells migration. Here, we identified the mechanism by which tumor-intrinsic Yap deletion resulted in HCC migratory inhibition. Yap was greatly upregulated in HCC and its expression promoted the cells migration. Functional studies found that knockdown of Yap induced JNK phosphorylation which closely bound to the Bnip3 promoter and contributed to Bnip3 expression. Higher Bnip3 employed excessive mitophagy leading to mitochondrial dysfunction and ATP shortage. The insufficient ATP inactivated SERCA and consequently triggered intracellular calcium overload. As the consequence of calcium oscillation, Ca/calmodulin-dependent protein kinases II (CaMKII) was signaled and subsequently inhibited cofilin activity via phosphorylated modification. The phosphorylated cofilin failed to manipulate F-actin polymerization and lamellipodium formation, resulting into the impairment of lamellipodium-based migration. Collectively, our results identified Hippo-Yap as the tumor promoter in hepatocellular carcinoma that mediated via activation of cofilin/F-actin/lamellipodium axis by limiting JNK-Bnip3-SERCA-CaMKII pathways, with potential application to HCC therapy involving cancer metastasis.

1. Introduction

Hepatocellular carcinoma (HCC) is reported as the most common one in digestive cancers in the worldwide [1]. Due to the rapid progression of HCC, most patients with this disease are diagnosed at advanced stage. In advanced HCC cases, the 5-year survival rate is as low as 25–39%, and the recurrence rate is approximately 80% [2]. Several patients underwent operative resection, however, these patients still suffered from a poor prognosis [3]. Notably, some HCC patients with advanced stage have no chances for operation, and their overall survival period is less than one year [4]. It has been reported that recurrence and metastasis account for the high mortality of HCC patients [5]. Therefore, it is critical to identify the potential molecular mechanisms underlying the progression and metastasis in HCC.

The Hippo network is a major conserved growth suppressor that participates in organ size control during development and prevents tumor formation during adult homeostasis [6]. The central component of the Hippo pathway is the transcriptional co-activator Yes-Activated Protein (Yap). Yap binds to transcription factor partners driving a transcriptional programme that specifies cell growth, proliferation, apoptosis, migration and invasion [7–9]. However, the mechanism by which Yap regulates the cellular migration or invasion is incompletely understood.

Cancer cells migrating into lymph nodes or blood vessels to form metastases is vital for the progression of HCC [10]. In tumor progression, cancer cells can migrate as single cells or collectively as groups in

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a lamellipodium-based migration mode [11]. Under this condition, cellular membrane extension in lamellipodia is driven predominantly through F-actin polymerization [12]. A large array of actin binding proteins (ABPs) have been found to be the regulator of F-actin polymerization and lamellipodium formation [13]. Among them, cofilin is an indispensable controller [14,15]. Dephosphorylated cofilin augments the F-actin synthesis and actin filament extension, which assist the formation of lamellipodia. What remains unknown is whether cofilin and actin-driven lamellipodium is regulated by Yap, and if so, what molecular links Yap to cofilin.

Cellular migration involves drastic structural changes, a process that demands high levels of energy and fully functional mitochondria [16] whose quality and quantity are balanced by mitophagy [17,18]. Our previous study has suggested that mitophagy could regulate the endothelial migration via modification of F-actin homeostasis [19]. Moreover, excessive mitochondrial damage such as mitochondrial fission would lead to the collapse of F-actin and lamellipodium [20,21]. These information indicate the possible relationship between mitochondria and lamellipodium-based migration. Given the available evidences linking Yap and mitochondria [22,23], we therefore want to know whether mitochondria, especially mitophagy, is the bridge connecting upstream Yap and downstream cofilin/F-actin. If so, what signals are responsible for mitophagy and cofilin/F-actin.

Apart from mitochondria, cellular migration also needs moderate intercellular calcium ($[Ca^{2+}]i$) concentration [24]. The excessive $[Ca^{2+}]i$ elevation would impair the cellular migration via activation of Ca/calmodulin-dependent protein kinases II (CaMKII) [25]. The CaMKII has the ability to phosphorylate cofilin [26]. Phosphorylated cofilin is an inactivate form without the ability to assembly F-actin and promote lamellipodium formation. Our previous study [27] has reported that the $[Ca^{2+}]i$ balance is highly dependent on the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity which uptakes 92% $[Ca^{2+}]i$ back to endoplasmic reticulum in the resting state. Meanwhile, SERCA is a Ca-ATPase and its activity is heavily relied on the cellular ATP production. Considering the decisive action of mitophagy in ATP production [28], we therefore ask whether mitophagy handles cofilin via SERCA-mediated $[Ca^{2+}]i$ imbalance and CaMKII activation. Thus, this study is undertaken to establish the regulatory effect of Yap on HCC migration, particularly focusing on the mitophagy-SERCA-CaMKII pathways and cofilin/F-actin/lamellipodium axis.

2. Methods

2.1. Patients and specimen selection

The present study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals, which was published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the guidelines of the Ethics Committee of Chinese PLA (People's Liberty Army) General Hospital, Beijing, China. The all experimental protocol was approved by Ethics Committee of Chinese PLA (People's Liberty Army) General Hospital, Beijing, China. In all, 6 samples of liver cancer were obtained from the department of oncology, PLA general hospital cancer center. Meanwhile, 6 samples of normal liver tissues that were obtained from patients who underwent surgery were included in this study. The informed consent forms, including blood sample collection consent and operation consent were obtained from all individual participants included in this study.

2.2. Cell culture

The liver cancer cell lines HepG2 and normal live cell lines (L02) used in the present study were obtained from the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), penicillin G (100 U/mL) and streptomycin (100 g/mL) and were maintained in monolayer culture at 37 °C in humidified air with 5% CO_2.

2.3. The qPCR and RNA interference

The qPCR assay was carried out according to our previous study [29]. The primers used for polymerase chain reaction were as follows: Bnip3, forward, TCCAGCCTCGGTTTCTATTT and reverse, AGCTCTTGGAGCTACTCCGT, YAP, forward, CCCAGACTACCTTGAAGCCA and reverse, CTTCCTGCAGACTTGGCATC, GAPDH, forward, GCGGGAAATCGTGCGTGAC and reverse, CGTCATACTCCTGCTTGCTG. Quantification of gene expression was performed using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) with SYBR Green (TransGen Biotech Co., Ltd., China). The mRNA levels were determined by qRT-PCR in triplicate for each of the independently prepared RNAs and were normalized to the levels of GAPDH expression.

The shRNA or scrambled shRNA control specific against the expression of Yap was constructed using a lentiviral shRNA technique (GeneChem), as described previously by our laboratory [30]. The shRNA was used to generate the stable cell line HepG2 without expression of Yap. To suppress the Bnip3, siRNA was used according to our previous study [19]. The siRNA targeting Bnip3 was purchased from Santa Cruz Biotechnology.

2.4. Western blot analysis

Cells were rinsed with cold PBS and harvested in lysis buffer [31]. Then, the extractions were obtained and then centrifuged at 14,000 rpm for 30 min. Twenty-five micrograms of protein was loaded per lane and separated by SDS-PAGE, then transferred to nitrocellulose membranes and blocked overnight in 5% skim milk. Then, the membrane was incubated with primary antibodies at 4 °C and subsequently incubated with a secondary antibody for 2 h at room temperature. The primary antibodies for the blots are as follows: p-CaMKII (1:1000, Cell Signaling Technology, Inc.), p-cofilin (1:1000, Abcam plc), CaMKII (1:1000, Cell Signaling Technology, Inc.), cofilin (1:1000, Cell Signaling Technology, Inc.), Beclin1 (1:1000, Cell Signaling Technology, Inc.), LC3II (1:1000, Cell Signaling Technology, Inc.), ATG5 (1:1000, 1:1000, Abcam plc), Yap (1:1000, Cell Signaling Technology, Inc.), F-actin (1:1000, 1:1000, Abcam plc), G-actin (1:1000, 1:1000, Abcam plc), p-JNK (1:1000, Cell Signaling Technology, Inc.), JNK (1:1000, Cell Signaling Technology, Inc.), Parkin (1:1000, Cell Signaling Technology, Inc.), p-Parkin (1:1000, Cell Signaling Technology, Inc.), Bnip3 (1:1000, Cell Signaling Technology, Inc.), p62 (1:1000, 1:1000, Abcam plc) and SERCA (1:1000, Cell Signaling Technology, Inc.)

2.5. Detection of Ca^{2+} concentration and SERCA activity

Intracellular Ca^{2+} $[Ca^{2+}]i$ was measured using the calcium-dependent fluorescent dye Fura-2 as our previous study described [27]. Briefly, 5 μL of Fura-2-acetoxymethyl ester (AM; 10 μM; Life Technologies, Carlsbad, CA, USA) was applied in the medium for 30 min [27]. The Fura-2-loaded cells were then observed under confocal microscope (Olympus).

The calcium mapping was used to quantify the calcium intensity according to our previous study [32]. Fluorescence intensity of Furo-2 was measured by excitation wavelengths of 340 and emission wavelengths of 500. Data (F/F0) were obtained by dividing fluorescence intensity (F) by (F0) at resting level (t = 0) which was normalized by control groups.

For SERCA activity assay, cells after treatment were harvested to isolate the total proteins. The protein concentration of the supernatant was determined using the BCA method. Sample protein concentrations were adjusted to 1000 μg/mL. The proteins were incubated in a 30 °C water bath for 10 min and 20 μL ATP was added. Thirty seconds later, 20 μL of the sample was added. After mixing for 45 s, the OD values were measured [33].