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Original Article

Mitochondrial fission forms a positive feedback loop with cytosolic calcium signaling pathway to promote autophagy in hepatocellular carcinoma cells

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ABSTRACT

Both mitochondrial morphology and the level of cytosolic calcium $[Ca^{2+}]c$ are actively changed and play critical roles in a number of malignancies. However, whether communications existed between these two processes to ingeniously control the malignant phenotype are far from clear. We investigated the reciprocal regulation between mitochondrial fission and cytosolic calcium signaling in human hepatocellular carcinoma (HCC) cells. Furthermore, the underlying molecular mechanisms and the synergistic effect on autophagy were explored. Our results showed that mitochondrial fission increased the $[Ca^{2+}]c$ and calcium oscillation in HCC cells. We further found that mitochondrial fission-mediated calcium signaling was dependent on ROS-activated NF-κB pathways, which facilitated the expression of STIM1 and subsequent store-operated calciumentry. Additionally, we also demonstrated that increase in $[Ca^{2+}]c$ promoted mitochondrial fission by up-regulating expression of Drp1 and FIS1 via transcription factors NFATC2 and c-Myc, respectively. Moreover, the positive feedback loop significantly promoted HCC cell global autophagy by Ca^{2+}/CAMKK/AMPK pathway. Our data demonstrate a positive feedback loop between mitochondrial fission and cytosolic calcium signaling and their promoting role in autophagy of HCC cells, which provides evidence for this loop as a potential drug target in tumor treatment.

Introduction

In a majority of cell types, mitochondria continually change their shape through the combined actions of fission and fusion in response to cellular energy demands and environmental challenges [27]. These activities determine the balance between mitochondrial energy production and cell death programs. Recently, cumulative evidence is beginning to reveal the close links between cancers and unbalanced mitochondrial dynamics [12]. We and others have shown that the expression of mitochondrial fission/fusion proteins such as Drp1, MFN1 and MFN2 is dysregulated in human cancers of liver, lung, bladder and breast and the disruption of mitochondrial network exhibits a considerable effect on the survival of cancer cells [7,20,35,41,43].However, to date, the mechanism of how mitochondrial dynamics is regulated in tumor cells is far from clear.

Just like mitochondrial dynamics, the ubiquitous cellular calcium signaling is also continually changed in response to different intracellular or extracellular stimulus in the forms of transient elevation, highly localized sparks, or calcium oscillations. These changes can be “decoded” by the cells, which allow the ubiquitous

Abbreviations: Drp1, dynamin 1-like; ER, endoplasmic reticulum; FIS1, fission, mitochondrial 1; IHC, immunohistochemistry; LMNB1, lamin B1; MFN1, mitofusin 1; MFN2, mitofusin 1; NFATC2, nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; OPA1, optic atrophy 1; Orai1, ORAI calcium release-activated calcium modulator 1; ROS, reactive oxygen species; SOCE, store-operated calcium entry; STIM1, stromal interaction molecule 1.

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calcium signal to specifically regulate cellular processes and thus impact nearly every aspect of cellular life. Although significant information about the role of calcium in cell fate is already well-established, the mechanism of how calcium signaling is regulated in tumor cells remains poorly understood.

Considering the situation that the changes of mitochondrial dynamics and cytosolic calcium may occur at the same time and location, we raised a question whether there may be a feedback regulation loop between mitochondrial fission and cytosolic calcium signaling, which coordinately regulate the biological processes of tumor cells, such as autophagy. Several clues list as below. First, it has been reported that mitochondrial fission increases cellular reactive oxygen species (ROS) in types of cells, including liver [40]and heart cells [19]. ROS accumulation, in turn, has been reported to affect several components involved in Ca^{2+} homeostasis and thus alters multiple intracellular pathways [2]. Second, several studies have reported that cytoplasmic Ca^{2+} elevation induces fragmented mitochondria and elevated Drp1 activity or expression in neurons, cardiomyocytes or liver cells [13,33,39], suggesting that cytosolic Ca^{2+} signaling may be involved in the regulation of mitochondrial dynamics. Third, our previous study have shown that the elevated mitochondrial fission stimulates a global autophagy but not specific mitophagy in HCC cells, suggesting that global signaling such as calcium may be involved in autophagy mediated by increased mitochondrial fission.

In the present study, we systematically investigated the crosstalk between mitochondrial fission and cytosolic calcium signaling and also their combined roles in autophagy. More importantly, the underlying molecular mechanisms were explored. Our study further facilitates our understanding of the pathological roles played by mitochondrial dynamics and provides strong evidence for novel strategy targeting proteins of mitochondrial fission machinery in treatment of human tumor.

Materials and methods

Cell culture and tissue samples collection

Human HCC cell lines Bel7402 and Huh-7 were from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). MHCC97L were from Liver Cancer Institute, Fudan University, Shanghai, China [26,38]. The three HCC cell lines were authenticated using short tandem repeat DNA testing by the FMMU Center for DNA Typing in 2016. HCC cells were routinely cultured. The same panel of HCC tissue samples and clinical data were described in our previous study [18].

Knockdown and forced expression of target genes

For the generation of shRNA expression vectors, a small hairpin RNA (shRNA) containing specific sequences targeting the human Drp1 or MFN1 mRNA sequence was cloned into the pSilencer™ 3.1-H1 puro vector (Ambion). A control shRNA was also cloned into the pSilencer™ 3.1-H1 puro vector, which was used as a silencing negative control. For the overexpression of Drp1, MFN1 or STIM1, the coding sequences of Drp1, MFN1 or STIM1 was amplified from cDNA derived from Bel7402 cells using primers listed in Supplementary Table 1 and cloned into the pcDNA™3.1 (+) vector (Invitrogen). All siRNAs were synthesized by GenePharma (Shanghai, China). The sequences of siRNA for *DRP1*, *FIS1* and *STIM1* are provided in Supplementary Table 1.

Antibodies and reagents

The primary antibodies used in this study and their working concentration were listed in Supplementary Table 2. Intracellular ROS scavenger NAC (*N*-acetylcysteine), Intracellular ROS inducer H_2O_2, intracellular calcium chelator BAPTA-AM, intracellular calcium inducter ATP, endoplasmic reticular Ca^{2+}-ATPase inhibitor TG (thapsigargin), NF-κB inhibitor Bay11-7082 and CAMKK inhibitor STO-609 were all purchased from Sigma-Aldrich (St. Louis, MO).

Mitochondrial network imaging by confocal microscopy

The fluorescent dye MitoTracker green FM (Invitrogen) were used to monitor mitochondrial morphology in living cells according to the manufacturer's instructions. Then cells were viewed with an Olympus FluoView™ FV 1000 laser-scanning confocal microscope. For morphometric analysis, the length of mitochondria was measured using the Image J software (NIH, Bethesda, MD). In addition, the number of mitochondria was counted and averaged in 50 cells per sample.

Measurement of cytosolic Ca^{2+} concentration

The fluorescent dye Fluo-4/AM (Invitrogen) were used to monitor cytosolic Ca^{2+} concentration in living cells according to the manufacturer's instructions. Briefly, HCC cells were seeded in 15-mm coverglass-bottom dish (NEST, Wuxi, China) and loaded with 4 μM Fluo-4/AM (Invitrogen), then washed with HBSS and followed by additional 30 min incubation at 37 °C to allow complete de-esterification of intracellular AM (acetoxymethyl) esters before fluorescence measurements. Cells were examined with a confocal laser scanning microscope (Olympus FluoView™ FV1000, Japan). Images were analyzed and quantitated using Olympus FluoView software. For measurement of Ca^{2+} oscillation, 10% FBS was added after 1 min of baseline recording, and images were recorded every 1 s for 10 min using the same confocal imaging system. Ca^{2+} oscillation frequency was determined by the time interval between two adjacent peaks (fluorescence intensity dramatically increased by more than 20%) over the 10 min recording period and expressed as the number of spikes per minute. For measurement of store-operated Ca^{2+} entry (SOCE), 500 nM TG (thapsigargin) were added in the Ca^{2+} free HBSS to deplete internal calcium stores. Ca^{2+} influx was induced by subsequent addition of 2 mM Ca^{2+}.

Detection of reactive oxygen species

Cellular reactive oxygen species (ROS) were detected by the fluorescent probe DCFH-DA (Beyotime Biotechnology, Shanghai, China) as previously described [18].

Quantitative reverse transcription PCR (qRT-PCR), western blot, immunofluorescence and immunohistochemistry (IHC)

RNA extraction, complementary DNA synthesis, qRT-PCR reactions were performed as previously described [17]. Primer sequences used in this study were provided in Supplementary information Table S1. HCC cell lines were processed for Western blot, immunofluorescence and IHC as previously described [17]. Quantification of IHC staining score was performed as previously described [17].

Serial deletions of gene promoter and site-directed mutagenesis

Promoter sequences of Drp1 and Fis1 were obtained from UCSC Genome Browser. Then pGL3-Basic vectors (Promega) inserted by truncated portions of *Drp1* or *FIS1* promoter were generally constructed by PCR amplification of selected regions with primers listed in Supplementary Table 1. Site-directed mutagenesis was performed using the Q5® Site-Directed Mutagenesis Kit (NEB) according to the manufacturer's instructions.

Luciferase reporter assay

4 μg of *Drp1* or *FIS1* promoter constructs were co-transfected with 50 ng of Renilla luciferase expressing control vector into 1×10^6 HCC cells. Transfected cells were cultured for 48 h. Cells were lysed and the Luciferase activates were determined using the Dual Luciferase Reporter Assay kit (Promega) according to the manufacturer's instructions. The relative light units were measured by a Luminoscan Ascent Microplate Luminometer (Thermo Scientific). The firefly luciferase activity corresponding to a specific promoter construct was normalized to Renilla luciferase activity.

Chromatin immunoprecipitation (ChIP) assay

A ChIP assay was performed using the EZ-ChIP kit (Millipore Corporation, Billerica, MA). Briefly, cell lysates were incubated with anti-NFATC2 or c-Myc or IgG antibodies. PCR reactions generated two 200–300-bp products from the regulatory region of the *Drp1* or *FIS1* gene. The sequences of primers are provided in Supplementary Table 1.

Evaluation of fluorescent LC3 puncta

HCC cells were transiently transduced with pcDNA3.1-GFP-LC3 followed by staining with MitoTracker Red FM (Molecular Probes, M22425). Autophagy was quantified by calculating the average number of GFP-LC3 puncta per cell in 5 high-power fields (HPF, 400×).

Statistical analysis

Experiments were repeated three times, where appropriate. Data represent mean ± SEM. SPSS 17.0 software (SPSS, Chicago, IL) was used for all statistical analyses and $P < 0.05$ was considered significant. Unpaired *t*-test was used for comparisons between two groups where appropriate. Correlations between measured variables were tested by Spearman rank correlation analysis.