Biochemical and Biophysical Research Communications 502 (2018) 30–42

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Speckle-type POZ protein suppresses hepatocellular carcinoma cell migration and invasion via ubiquitin-dependent proteolysis of SUMO1/sentrin specific peptidase 7

Piyou Ji [a], Shaohua Liang [b], Pengtao Li [a], Chengrong Xie [c], Jie Li [c], Kang Zhang [c], Xuqing Zheng [c], Min Feng [c], Qiu Li [c], Hui Jiao [c], Xiaoqin Chi [c], Wenxiu Zhao [c], Sheng Zhang [c], Xiaomin Wang [a, c, *]

[a] Department of Hepatobiliary Surgery, School of Clinical Medicine, Fujian Medical University, Fuzhou, Fujian, 350108, China
[b] Department of Human Anatomy, Histology and Embryology, School of Basic Medical Sciences, Fujian Medical University, Fuzhou, Fujian, 350108, China
[c] Fujian Provincial Key Laboratory of Chronic Liver Disease and Hepatocellular Carcinoma, Xiamen Translational Medical Key Laboratory of Hepatobiliary and Pancreatic Tumor, Zhongshan Hospital Xiamen University, Xiamen, 361004, China

ARTICLE INFO

Article history:
Received 9 May 2018
Accepted 16 May 2018
Available online 24 May 2018

Keywords:
SPOP
SENP7
Vimentin
HCC
Ubiquitination
Metastasis

ABSTRACT

Hepatocellular carcinoma (HCC) is associated with high metastatic potential and high mortality. Accumulating evidence has demonstrated that speckle-type POZ protein (SPOP) is a key adaptor molecule of ubiquitination. However, the molecular mechanism of SPOP-mediated ubiquitination in HCC metastasis remains obscure. In the present study, our results indicated that SPOP expression was significantly downregulated in HCC and was associated with tumor size, differentiation and metastasis. Cox regression model showed that low SPOP expression was a risk factor related to the prognosis of HCC patients. Loss- and gain-of-function assays demonstrated that SPOP inhibited HCC cell migration and invasion *in vitro*. Mechanisitically, co-immunoprecipitation and ubiquitination assays revealed that SPOP recognized and bound SENP7 and promoted its degradation via ubiquitin-dependent proteolysis. Analysis of immunohistochemistry showed that vimentin expression was correlated negatively with SPOP and positively with SENP7. These results implied that increased degradation of SENP7 by overexpression of SPOP decreased vimentin levels, which in turn attenuated HCC cell metastasis. Moreover, *in vivo* assays showed that SPOP overexpression also significantly suppressed liver and lung metastases. In summary, SPOP inhibits HCC cell metastasis via ubiquitin-dependent SENP7 proteolysis and may thus serve as a new opinion for the prevention of HCC metastasis.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and is ranked third in terms of cancer deaths worldwide, according to GLOBOCAN estimates [1]. About half of all liver cancer cases and liver cancer-related deaths occur in China [2]. Recent reports indicated that its aggressive migration and invasion characteristics are the main reasons for the high recurrence and mortality rates of HCC [3] and the main stumbling block to its effective treatment [4,5]. Clarifying the mechanisms for its aggressive migration and invasion thus represents a promising strategy for improving the prognosis of HCC.

Ubiquitination plays an important role in tumor migration and invasion by directly regulating the stability, activity, and localization of target proteins [6,7]. Recent reports have confirmed that speckle-type POZ protein (SPOP) can regulate the stability of multiple proteins and signaling transmission pathways via ubiquitination [6,8], and growing evidence suggests that SPOP functions as a key molecule in the development and progression of many tumors by regulating tumor-promoting/inhibiting molecules [6,9–17]. Specifically, SPOP promoted the progression of clear cell renal cell carcinoma by ubiquitination and degradation of important factors such as PTEN, DUSP7, Daxx, and Gli2, which are crucial for cell proliferation and apoptosis [10]. In contrast, SPOP also

* Corresponding author. Fujian Provincial Key Laboratory of Chronic Liver Disease and Hepatocellular Carcinoma, Xiamen Translational Medical Key Laboratory of Hepatobiliary and Pancreatic Tumor, Zhongshan Hospital Xiamen University, 201 Hubin South Road, Xiamen, Fujian Province, 361004, China.
E-mail address: wxm2203@xmu.edu.cn (X. Wang).

https://doi.org/10.1016/j.bbrc.2018.05.115
0006-291X/

influenced the Hedgehog/Gli2 signaling pathway via ubiquitination of Gli2 to suppress the progression of gastric and colorectal cancers [11,18], and inhibited the progression of prostate cancer by ubiquitination of ERG, EglN2, and c-Myc [12,15,17]. SPOP demonstrates great potential as a substrate-recognizing adaptor during ubiquitination, resulting in the degradation of different substrates to exert different functions, including tumor promotion and tumor inhibition. Although the function of SPOP has been widely studied, its role in HCC remains poorly understood. Although one report indicated that SPOP could downregulate Zeb2 mRNA to inhibit HCC cell metastasis [19], the involvement of SPOP-mediated ubiquitination in HCC cell migration and invasion remains elusive. We therefore focused on identifying the downstream substrates regulated by SPOP in HCC cell metastasis.

Post-translation modification of small ubiquitin-related modifier (SUMO) (SUMOylation) is a reversible process regulated by the sentrin/SUMO-specific proteases (SENPs), resulting in cleavage of the SUMO isopeptide from the modified proteins. SENP7 acts as a SUMO2/3-specific protease to cleave SUMO2 and SUMO3 [20–23], and has been shown to be involved in various biological processes [24–28]. SPOP modulates ubiquitin-dependent SENP7 proteolysis during cellular senescence [14]. The role of SENP7 in malignancies is poorly known. However one study showed that SENP7 promoted the expression of vimentin in breast cancer via the deSUMOylation of heterochromatin protein 1α [29], and vimentin is regarded a key executor molecule in tumor metastasis [30–33]. We therefore proposed the hypothesis that SPOP may regulate HCC cell migration and invasion by modulating SENP7 through the ubiquitin-proteasome pathway.

2. Materials and methods

2.1. Human HCC samples

All clinical samples and follow-up information for HCC patients were provided by the Chronic Liver Disease Biological Sample Bank, Department of Hepatobiliary Surgery, Zhongshan Hospital Xiamen University, with the patients' informed consent. All the procedures complied with the approved guidelines and were approved by the Ethics Committee (No: 20111008) of Zhongshan Hospital Xiamen University. Forty-four pairs of paraffin-embedded HCC and matched adjacent normal tissues and a further 74 pairs of matched tissues' mRNA from HCC patients were used in the experiments. Patient's survival was defined as the postoperative survival time or the time from surgery to final follow-up.

2.2. Immunohistochemistry and hematoxylin-eosin staining

Immunohistochemistry and hematoxylin-eosin staining were mainly performed as described previously [34,35], with some modifications according to the antibody instructions. For immunohistochemistry, paraffin-embedded sections (3 μm) were dewaxed and hydrated, subjected to antigen repair and blocking of endogenous peroxidase, and then incubated with primary antibodies overnight at 4 °C. The following steps were carried out using an Immunohistochemistry Kit (Maxin, China), according to the manufacturer's instructions. The primary antibodies used were SPOP (1:4000, clone 9B7.1, Millipore), SENP7 (1:3000, HPA027259-100 μl, Sigma-Aldrich), and vimentin (1:100, D21H3, CST), respectively. All samples were examined and confirmed by two expert pathologists in an independent and random manner. SPOP expression levels were quantified in 44 paired HCC and matched adjacent normal tissues by integrated optical density (IOD) values.

2.3. Western blot analysis

Proteins were extracted and their concentrations were measured. They were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride membranes (Millipore), and exposed by enhanced chemiluminescence (ECL, 1859698 and 1859701, Thermo), as described previously [10,14]. The quantification was made in western blot by integrated optical density (IOD) using Gelpro 32 software. The relative SPOP, SENP7 and vimentin protein levels were calculated by comparison to the IOD of β-Actin protein. The corresponding primary antibodies were SPOP (1:1000, 16750-1-AP, Protein-Tech, China), SENP7 (1:1000, NBP1-50027, Novus), vimentin (1:1000, D21H3, CST), and β-actin (1:1000, 0446WS, CMCTAG). Experiments were carried out in triplicate.

2.4. Quantitative real-time polymerase chain reaction

Reverse transcription polymerase chain reaction (PCR) and quantitative real-time PCR (qRT-PCR) were performed as described previously [34]. Briefly, RNA was extracted and reverse transcribed using a one-step kit (Invitrogen), and then used for qRT-PCR using a LightCycler96 (Roche). The peak of the melting curve was confirmed as the criterion for amplification specificity. Each sample was amplified using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous reference, and the data were analyzed by the ΔΔCT method. The primers are shown in Table 1. All the experiments were carried out in triplicate.

2.5. Migration and invasion assays

Based on previous procedures [35,36], the durations of the migration and invasion assays were adjusted based on the migration and invasive abilities of the HCC cells, but did not exceed 48 h. Briefly, HCC cells were plated in the upper chamber (Corning) with 8 μm holes at the bottom, filled with DMEM/high-glucose without fetal bovine serum (FBS, P160560, PAN Biotech). The same medium with 10% FBS was placed in the lower chamber. After 22 h, the cells on the bottom of the lower chamber were harvested and fixed with 4% paraformaldehyde (Solarbio, China), and the cells on the upper surface were wiped off and dyed with 0.5% crystal violet. The invasion assay was carried out as described above, except that the bottom of the upper chamber was coated with Matrigel. Five random microscopic fields per well were counted in a double-blind manner.

2.6. Cell proliferation assay

Cell proliferation was detected using a Cell Counting Kit-8 (CCK-8, KM672, Dojindo Laboratories, Japan) according to the manufacturer's protocol. Briefly, the cells were seeded onto 96-well plates at a density of 2×10^3 cells per well, and cultured for 1–5 days. During culture, 10 μl CCK-8 solution and 100 μl DMEM medium without FBS were added into the wells and incubated for 1 h. The resulting color was obtained at 450 nm using a microplate absorbance reader (Bio-Rad). All the experiments were carried out in triplicate.

2.7. Plasmid construction and lentivirus preparation

Over-expression plasmids were constructed according to the backbone of the PLV-C-HA vector for SPOP or pBoBi-CMV vector for SENP7, Myc-SPOP, SPOP-ΔM, and SPOP-ΔP. A SPOP-knockdown plasmid was constructed based on the PLKO.1-TRC-short hairpin RNA (shRNA) vector. The primers for the target genes and shRNA sequences are shown in Table 2. Lentivirus particles were packed