Biomedicine & Pharmacotherapy 93 (2017) 398–405

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com/en

Original article

SRPX2, an independent prognostic marker, promotes cell migration and invasion in hepatocellular carcinoma

Xiaobo Lin[a,1], Weiping Chang[a,1], Yuan Wang[b], Ming Tian[a], Zhaoxiang Yu[a,*]

[a] Department of General Surgery, The First Affiliated Hospital of Xi'an Medical University, Xi'an, Shaanxi Province 710077, China
[b] Department of Infectious Disease, The Second Affiliated Hospital of Xi'An Jiaotong University, Xi'an, Shaanxi Province 710004, China

ARTICLE INFO

Article history:
Received 10 April 2017
Received in revised form 18 June 2017
Accepted 20 June 2017

Keywords:
SRPX2
HCC
Tumor metastasis
FAK/AKT pathway
MMP2/9

ABSTRACT

Sushi repeat-containing protein X-linked 2 (SRPX2), a novel chondroitin sulfate proteoglycan, is overexpressed in human cancer. Recent studies have reported that SRPX2 overexpression is observed in gastrointestinal cancer, and promotes migration and invasion of cancer cells. While, the clinical significance and biological function of SRPX2 remain rarely known in hepatocellular carcinoma (HCC). Here, we found that the levels of SRPX2 in HCC tissues were notably overexpressed compared to non-cancerous specimens. Accordingly, the levels of SRPX2 were obviously up-regulated in HCC cells compared with LO2 cells. The positive expression of SRPX2 was prominently correlated with venous infiltration and advanced TNM tumor stage. Furthermore, SRPX2 expression acted as an independent prognostic marker for HCC patients. SRPX2 knockdown prominently inhibited the invasion and migration of HCCLM3 cells, while SRPX2 restoration enhanced these cellular biological behaviors of Hep3 B cells *in vitro*. Moreover, SRPX2 knockdown suppressed pulmonary metastasis of HCCLM3 cells in nude mice. Mechanically, SRPX2 knockdown reduced the levels of phosphorylated focal adhesion kinase (p-FAK), p-AKT, matrix metallopeptidase 2 (MMP2) and MMP9 in HCCLM3 cells. In turn, SRPX2 overexpression promoted the activation of FAK/AKT pathway and increased MMP2/9 expression in Hep3 B cells. Thus, SRPX2 contributes to migration and invasion of HCC cells probably by targeting FAK/AKT pathway-mediated MMP2/9 expression. SRPX2 potentially acts as an independent prognostic predictor and a drug-target for HCC patients.

1. Introduction

Hepatocellular carcinoma (HCC) is the commonest malignancy primarily initiated from liver and is usually presented in liver cirrhosis patients [1]. The incidence of HCC in male is notably higher than that in female [2]. Though the strategies of diagnosis and therapy for HCC have been remarkably improved in the last several decades, the prognosis of HCC remains poor [3]. Therefore, it is important to disclose a critical molecular biomarker for predicting poor clinical response and outcome of HCC patients.

Sushi repeat-containing protein X-linked 2 (SRPX2) is confirmed as a secreted protein that regulates synapse density in dissociated hippocampal neurons [4]. The mutation of SRPX2 is responsible for the disorders of Rolandic and Sylvian speech areas [5]. In human cancer, SRPX2 is firstly described as a sushi-repeat protein that is overexpressed in leukemia. Recently, SRPX2 expression has been demonstrated to be up-regulated in glioblastoma [6], colorectal cancer [7,8] and gastric cancer [9,10]. In these malignancies, SRPX2 commonly functions as an oncogene to promote the proliferation, migration and invasion of cancer cells [6–10]. However, the clinical significance and biological role of SRPX2 in HCC remain largely unknown.

In this study, we demonstrate that SRPX2 is overexpressed in HCC tissues. HCC patients who have positive expression of SPRX2 show malignant clinical characteristics and reduced survival. Our results show that SRPX2 promotes migration and invasion of HCC

Abbreviations: HCC, hepatocellular carcinoma; SRPX2, sushi repeat-containing protein X-linked 2; FAK, focal adhesion kinase; IHC, immunohistochemistry; qRT-PCR, quantitative real-time polymerase chain reaction; MMP 2, matrix metallopeptidase 2; HE, hematoxylin-eosin.

* Corresponding author at: Department of General Surgery, The First Affiliated Hospital of Xi'an Medical University, 48 Fenghao West Road, Xi'an, Shaanxi Province 710077, China.
E-mail address: yuzhaoxiang_jgsy@163.com (Z. Yu).

[1] Contributed equally.

http://dx.doi.org/10.1016/j.biopha.2017.06.075
0753-3322/

cells. Moreover, Focal adhesion kinase (FAK)/AKT pathway is recognized as a potential target of SRPX2 in HCC cells.

2. Materials and methods

2.1. Clinical samples

Sixty-eight clinical specimens including HCC and matched tumor-adjacent tissues were obtained from patients, who underwent hepatectomy and were histologically diagnosed as HCC in the First Affiliated Hospital of Xi'an Medical University. Patients who received immunotherapy, chemotherapy or radiotherapy before surgical treatment were excluded. Informed consent was signed by each patient before clinical specimens being collected and used. All specimens were formalin-fixed and paraffin-embedded for further investigation. The protocols involved in clinical specimens in this study were permitted by the Research Ethics Committee of Xi'an Medical University.

2.2. Cell culture and transfection

Human HCC cell lines including Hep3B, MHCC97L, MHCC97H and HCCLM3, and the human immortalized normal hepatocyte cell line (LO2) (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone) and antibiotics (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C in a humidified incubator containing 5% CO2.

SRPX2 shRNA and non-targeting (NT) shRNA, SRPX2 overexpression plasmid (pcDNA3.1-SRPX2) and empty vector were designed and synthesized by GenePharma (Shanghai, China). All vectors were then transfected into HCC cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.3. Immunohistochemistry (IHC)

The HCC tissues that were previously formalin-fixed and paraffin-embedded were sliced into 4 μm sections, which underwent deparaffination and then rehydration. Antigen retrieval, destruction of endogenous peroxidase activity and blocking with 10% skim milk were subsequently performed. Sections were incubated with SRPX2 primary antibody (ab91584, Abcam, Cambridge, MA, USA) overnight at 4 °C. The slides were subsequently incubated with peroxidase conjugated secondary antibody (ZSGB BIO, Beijing China) and DAB solution according to the manufacturer's instructions. The sections were counterstained with hematoxylin followed by dehydrating and mounting. SPRX2 staining was evaluated under a light microscope at 400 × magnification. Staining intensity was scored manually by two independent experienced pathologists as no staining = 0, weak staining = 1, moderate staining = 2, and strong staining = 3. Tumor cells in five fields were randomly selected and scored to get the average percentage of positively stained cells (0–100%). Staining quantity was graded as <25% = 1, 25%-75% = 2, and >75% = 3. The final IHC score was calculated by multiplying the intensity score with the quantity score. IHC score ≥3 was considered as positive expression of SRPX2.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacture's protocols. The first strand cDNA was synthesized using a Tianscript RT kit (Tiangen biotech, Beijing, China). PCR amplification for SRPX2 mRNA was performed with the SYBR Premix Ex Taq™ Kit (Takara, Shiga, Japan) in ABI 7300 system (Applied Biosystems, Foster City, CA, USA). GAPDH was employed as a reference gene to normalize the expression of SRPX2 mRNA. The primers for SRPX2 and GAPDH were synthesized and purchased from Sangon Biotech (Shanghai, China).

2.5. Migration and invasion assay

SRPX2 modified HCC cells were seeded onto 35 mm dishes until the fusion reaching above 90%. 100 μL pipette tip was used to make a wound at the middle of the dishes. Remnant cells were washed away by PBS. Serum-free DMEM medium was used to culture cells for 48 h. The healing of the wound was observed under the inverted microscope. Transwell chambers (coring costar, Cambridge, MA, USA) were employed to evaluate the invasive ability of HCC cells. HCC cells were resuspended in serum free DMEM medium and subsequently seeded in the upper chambers. To induce the invasion of HCC cells, the lower chambers were filled with 600 μL DMEM supplemented with 20% FBS. 48 h after cell seeding, HCC cells that invaded through the membranes (the membranes were covered with 70 μL matrigel) were stained with crystal violet for cell counting under the microscope.

2.6. Western blotting

Total cell lysates were extracted with RIPA Lysis Buffer and PMSF (Beyotime Co., China), and quantified with a BCA protein assay kit (Pierce, Bonn, Germany). Proteins were separated by 10% SDS-polyacrylamide gels and subsequently transferred to PVDF membranes (Millipore, Bedford, MA, USA). After blocking with 5% non-fat milk, the membranes were incubated with primary antibodies including SRPX2 (Abcam), p-FAK (Tyr397, #8556, Cell Signaling Technology, Beverly, MA, USA), FAK (#13009, Cell Signaling Technology), p-AKT (Ser473, #4060, Cell Signaling Technology), AKT (#9272, Cell Signaling Technology), matrix metallopeptidase 2 (MMP2, #87809, Cell Signaling Technology), MMP9 (#13667, Cell Signaling Technology). The secondary antibodies were obtained from Cell Signaling Technology (#7074 and #7076). GAPDH (sc-47724, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a loading control. The protein bands on the membranes were detected with ECL Advance Western detection reagents (GE Healthcare, Buckinghamshire, UK) and visualized with ChemiDoc XRS plus system (BIORAD, Hercules, CA, USA).

2.7. Experimental mouse model

BALB/c nude mice aged 4 weeks old were used to establish a model of pulmonary metastasis. HCCLM3 cells that were transfected with NT shRNA or SRPX2 shRNA were intravenously injected through the tail vein of nude mice. Nine weeks later, the lungs were harvested after euthanasia. Formalin-fixed and paraffin-embedded lung tissues were sectioned and stained for hematoxylin-eosin (H&E) [11], and the metastatic nodules were counted. All animal experiments were approved by the Ethics Committee of Xi'an Medical University.

2.8. Statistical analysis

Data were presented as Mean ± SD and analyzed by GraphPad Prism 5 software (GraphPad Software, Inc, San Diego, CA, USA). Chi-squared test was employed to explore the association between two variables. The Student's *t*-test and ANOVA were carried out to analyze continuous variable. The prognostic significance of SRPX2 was determined using the Kaplan-Meier method, Log-rank test