Cancer Letters 388 (2017) 177–186

Contents lists available at ScienceDirect

Cancer Letters

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

Original Article

STC2 as a novel mediator for Mus81-dependent proliferation and survival in hepatocellular carcinoma

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ARTICLE INFO

Article history:
Received 1 October 2016
Received in revised form
29 November 2016
Accepted 30 November 2016

Keywords:
Mus81
Hepatocellular carcinoma
STC2
Proliferation
Apoptosis

ABSTRACT

Methyl methansulfonate and UV sensitive gene clone 81 (Mus81) is a critical DNA repair gene that has been implicated in development of several cancers including hepatocellular carcinoma (HCC). However, whether Mus81 can affect proliferation and survival of HCC remains unknown. In the present study, we demonstrated that the knockdown of Mus81 was associated with suppressed proliferation and elevated apoptosis of HCC cells *in vitro* and *in vivo*. Multilayered screenings, including DNA microarray, high content screen, and real-time PCR validation, identified STC2 as a proliferation-facilitating gene significantly down-regulated in HCC cells upon Mus81 knockdown. STC2 expression was also closely correlated to Mus81 expression in HCC tissues. More importantly, the restoration of STC2 expression recovered the compromised cell proliferation and survival in Mus81 depleted HCC cells. Furthermore, Mus81 knockdown was associated with the activation of APAF1, APC, and PTEN pathways and concurrent inhibition of MAPK pathway through decreasing STC2 expression. In conclusion, Mus81 knockdown suppresses proliferation and survival of HCC cells likely by downregulating STC2 expression, implicating Mus81 as a therapeutic target for HCC.

Introduction

Hepatocellular carcinoma (HCC) ranks the fifth most common cancer worldwide and becomes the third leading cause of cancer-related mortality [1]. Unfortunately, the incidence of this deadly disease is still increasing in many countries even including the United States [1,2]. Despite a great improvement in diagnostic technology and surgical treatment, the long-term survival of this malignancy is still unsatisfactory because only around 40% of HCC patients are eligible for potentially curative treatments (hepatic resection and liver transplantation) [3]. To address this situation, great efforts have been made to elucidate the molecular mechanisms underlying HCC development and progression in order to identify targets for HCC treatment [4]. Studies have uncovered several signaling pathways such as mitogen-activated protein kinase (MAPK), vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR) critically involved in proliferation, apoptosis, migration and angiogenesis in HCC cells [4,5]. Other mechanism such as autophagy has also been recently correlated to the carcinogenesis of HCC [5]. These mechanistic studies have led the multikinase inhibitor sorafenib to the clinic, which is now the standard cure for patients with advanced-stage HCC [3–6]. However, the benefits obtained from sorafenib are still limited (only prolongs nearly three months overall survival) [3,4,6], raising a pressing need for discovery of novel and effective therapy [4,5].

Many studies have shown that DNA repair genes are significantly correlated to the susceptibility and the prognosis of HCC [7,8]. Targeting these DNA repair genes may serve as a potential therapeutic strategy for HCC [7,9,10]. Methyl methansulfonate and UV sensitive gene clone 81 (Mus81) is a highly conserved gene across the species and encodes a structure-specific DNA endonuclease, which resolves holliday junctions (HJs) and plays a critical role in the repair of double-strand breaks (DSBs) of DNA and

Abbreviations: Mus81, methyl methansulfonate and UV sensitive gene clone 81; HCC, hepatocellular carcinoma; STC2, Stanniocalcin 2; APC, adenomatous polyposis coli; APAF1, apoptotic protease activating factor 1; PTEN, phosphatase and tensin homolog; HCS, high content screen; PCR, polymerase chain reaction; shRNA, short hairpin RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium bromide; DMSO, dimethyl sulfoxide; H&E, hematoxylin and eosin; IHC, immunohistochemistry; TUNEL, terminal deoxynucleotidyl-transferase-mediated d-UTP-biotin nick end-labeling.

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http://dx.doi.org/10.1016/j.canlet.2016.11.039
0304-3835/

maintenance of chromosomal integrity [11,12]. Mus81 is usually regarded as a potent tumor suppressor and its downregulation has been associated with several types of cancers [13–16]. Our previous study also reported the decrease of Mus81 expression in human HCC tissues, which is correlated to the progression and prognosis of this malignancy [17]. However, a number of recent studies suggested Mus81 inhibition as a novel approach to sensitize HCC and breast cancer cells to chemotherapeutic agents [18,19]. The latest study even revealed the effects of Mus81 knockdown not only on chemosensitivity but also on cell proliferation of serous ovarian cancer, although the underlying molecular mechanism is still unclear [20].

However, whether Mus81 modulates cell proliferation and apoptosis of HCC remains unknown. Therefore, we carried out the present study to explore the biological consequences of Mus81 knockdown by lentivirus-mediated short hairpin RNA (shRNA). Cell proliferation and apoptosis of human HCC cell lines were examined through a series of *in vitro* and *in vivo* experiments. Furthermore, the genes and signaling pathways associated with Mus81 were also investigated by DNA microarray combined with high content screen and polymerase chain reaction (PCR) pathway array. The results were further validated in HCC tissues, cells and xenograft tumors.

Materials and methods

Cell culture and reagents

SMMC-7721, SK-HEP-1, Bel-7402, and Bel-7404 HCC cell lines were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (SIBS, CAS). HepG2 and Hep3B HCC cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). All these cell lines were cultured in high glucose DMEM containing 10% fetal bovine serum at 37 °C in a humidified incubator (SANYO, Osaka, Japan) with 5% CO_2. The primary antibody against Mus81, Stanniocalcin 2 (STC2), adenomatous polyposis coli (APC), apoptotic protease activating factor 1 (APAF1), AKT, phosphorylated AKT (Ser473), β-catenin, GAPDH, Histone H3, MAPK3, phosphorylated MAPK3 (Tyr204), and phosphatase and tensin homolog (PTEN) were purchased from Abcam (Cambridge, UK). The primary antibody against Caspase-9 and the cleaved Caspase-9 were purchased from Cell Signaling Technology (Danvers, MA).

Lentivirus-mediated short hairpin RNA (shRNA)

GV248-GFP-Lentivirus shRNA vector was purchased from GeneChem (Shanghai, China). The shRNA sequence targeting Mus81 or STC2 were all chosen from 3 candidate sequences (data not shown) and these sequences and the negative control sequences (scramble sequences not targeting any known gene) were listed in Supplementary Table S1. The lentivirus was packaged, purified and infected as described previously [18]. HCC cells infected with lentivirus containing shRNA#1 sequence targeting Mus81/STC2 and the negative control sequence were named as shMus81/shSTC2 and shCtrl respectively.

Gene expression vector

Full-length human Mus81 and STC2 cDNA were obtained by polymerase chain reaction (PCR) amplification as described previously [21] and mutated Mus81 cDNA sequence was generated by PCR-mediated site-directed non-sense mutagenesis to avoid the knockdown by shRNA targeting Mus81 (Supplementary Fig. S1). Then mutated Mus81 and STC2 expression vector were generated respectively by subcloning mutated Mus81 or STC2 cDNA into the GV143 plasmid (purchased from GeneChem).

Western blot

Total protein was extracted and separated by SDS-PAGE and then transferred onto PVDF membrane (Merck Millipore). The blotted membranes were incubated with primary antibody against Mus81, STC2, APC, APAF1, AKT, phosphorylated AKT (Ser473), β-catenin, MAPK3, phosphorylated MAPK3 (Tyr204), PTEN, Caspase-9 and the cleaved Caspase-9 and then the corresponding secondary antibody in order. GAPDH protein was also determined as a loading control as well as a cytoplasmic protein marker. Nuclear protein Histone H3 was used as a nuclear protein marker [22].

MTT assay

Cells were seeded in 96-well plates at initial density of 2×10^3 per well. At each time point, cells were stained with 10 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL, Sigma) for 4 h at 37 °C in the dark. After removing the supernatant, formazan crystals formed were dissolved in 100 μL dimethyl sulfoxide (DMSO) and the absorbance was measured at 490 nm. All experiments were carried out in quintuplicate.

Colony formation assays

Cells were plated in 6-well plates (8×10^2 cells/well) and cultured for 14 days. The colonies were stained with 500 μL Giemsa dye (Chemicon International, Temecula, CA) for 30 min after fixed with 4% paraformaldehyde for 30 min. The sizes of cell colonies were also observed by a light microscope (Nikon, Tokyo, Japan).

Flow cytometric analysis

Cell apoptosis rate was analyzed by Annexin V-FITC/PI (propidium iodide) stained fluorescence-activated cell sorter (FACS). Briefly, every kind of HCC cell were harvested, washed twice with cold phosphate buffered saline (PBS) and resuspended at 1×10^6 cells/ml in 100 μL 1× binding buffer (eBioscience, San Diego, CA). Annexin V-FITC (5 μL, eBioscience) and PI (5 μL, eBioscience) were added to the cell suspension (100 μL) followed by incubation at room temperature for 15 min in the dark and then mixed with 400 μL 1× binding buffer. All samples were analyzed by a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) using Cell Quest Research Software (BD Biosciences). The cells in early or late-stage apoptosis (Annexin V-FITC positive) were all defined as apoptotic cells. Apoptosis rate was calculated as apoptotic cells/total cells. And those HCC cells infected with lentivirus containing negative control shRNA or empty vector were analyzed as control. To analyze the cell cycle distribution of HCC cells, these cells were collected and fixed in cold 70% ethanol overnight at 4 °C. After washing twice with PBS, the cells were subsequently stained with PI (50 μg/mL) and RNase A (100 μg/mL) for 1 h and subjected to flow cytometric analysis. HCC cells infected with lentivirus containing negative control shRNA were used as control.

Mouse xenograft tumor model and bioluminescence imaging

Six to eight weeks old nude mice were obtained from Tongji University Experimental Animal Center (Shanghai, China). SMMC-7721-shCtrl-Luc and SMMC-7721-shMus81-Luc cells were inoculated subcutaneously on the right upper flank regions of these mice (1×10^7 cells/mouse). After inoculation, the body weights and the xenograft tumor growth of these mice were measured every other day and the tumor volumes (V) were calculated using the formula: $V = a \times b^2/2$, where a and b are the largest and smallest tumor diameter, respectively. To determining the viability of SMMC-7721 cells inside the xenograft tumors, bioluminescence imaging was performed in 26 days after inoculation. For bioluminescence imaging, mice were anesthetized by 0.7% pentobarbital sodium (100 μL/10 g bodyweight) i.p. and received luciferin (Sigma, 150 mg/kg bodyweight) i.p. in a volume of 100 μL isotonic saline solution in 5 min after anesthesia. After 15 min, bioluminescence imaging was performed by measuring luciferase activity *in vivo* using a Peltier cooled charged-coupled device camera (NightOWL LB 983; Berthold, Bad Wildbach, Germany) with exposure time for 2 min. All the mice were sacrificed immediately after bioluminescence imaging and the xenograft tumors were dissected out, weighed up and made into 4 μm thick sections for hematoxylin and eosin (H&E) staining, immunohistochemistry (IHC) and terminal deoxynucleotidyl-transferase-mediated d-UTP-biotin nick end-labeling (TUNEL) assay. Animal use and care followed institutional guidelines established by the Guangzhou Red Cross Hospital Institutional Animal Care and Use Committee.

IHC and TUNEL assay

The expression of Mus81, Ki-67, STC2, APC, APAF1, PTEN, phosphorylated MAPK3 (Tyr204), and apoptosis level in xenograft tumors were detected by IHC and TUNEL assay respectively as described previously [18].

DNA microarray and data analysis

Total RNA was extracted from SMMC-7721-shCtrl and SMMC-7721-shMus81 cells using TRIzol reagent (Invitrogen, Frederick, MD). The quality of RNA was checked using the Nanodrop 8000 spectrophotometry (Thermo Scientific, Waltham, MA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Fifty nanograms of the total RNA was converted to cDNA and amplified according to the manufacturer's instructions for the Affymetrix GeneChip™ System (Affymetrix, Inc., Santa Clara, CA). The double stranded cDNA was used as a template for an *in vitro* transcription reaction for the synthesis of biotinylated cRNA using a GeneChip 3′ IVT Express Kit (Affymetrix, Inc.). The labeled cRNA probes were then fragmented and hybridized onto the GeneChip® PrimeView™ Human Gene Expression Array according to manufacturer's instructions. After hybridization, the arrays were washed and stained in GeneChip Fluidic station 450 (Affymetrix, Inc.). After washes, probe arrays were scanned in GeneChip Scanner 3000 (Affymetrix, Inc.) using GeneChip™ Operating System v1.3 software (GCOS, Affymetrix, Inc.). Differentially expressed genes (DEGs) between SMMC-7721-shCtrl and SMMC-7721-shMus81 cells were identified as expression fold change was >2 and P value was <0.05. Subsequently, DEGs were put into Gene Ontology (GO)