RASSF7 promotes cell proliferation through activating MEK1/2-ERK1/2 signaling pathway in hepatocellular carcinoma

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Abstract: The Ras-association domain family (RASSF) proteins have been involved in many important biological processes. RASSF7 is recently reported to be up-regulated in several types of cancer. However, the function of RASSF7 remain unknown in human cancers. To explore the role of RASSF7 in hepatocellular carcinoma (HCC) cells proliferation and molecular mechanism. RASSF7 expression was examined using public database TCGA, qRT-PCR and Western blot. The correlation between RASSF7 and clinicopathological features was measured. Overexpression and silencing of RASSF7 were performed to measure the impact on HCC cell proliferation, cell cycle and apoptosis. Furthermore, the molecular mechanism of MEK1/2-ERK1/2 signaling pathway regulation by RASSF7 was explored. RASSF7 was significantly up-regulated in HCC tissues and cell lines, and correlated with AFP, poor tumor histology and T stage. Overexpression of RASSF7 promoted HCC cell proliferation, driven G1-S phase cell cycle transition and inhibited apoptosis. Knockdown of RASSF7 suppressed cell growth, induced G1-S phase cell cycle arrest and cell apoptosis. Furthermore, our findings also demonstrated that RASSF7 promoted HCC cell proliferation through activating MEK1/2-ERK1/2 signaling pathway. Taken together, this study provides a novel evidence for clinical significance of RASSF7 as a potential biomarker, and demonstrates that RASSF7- MEK1/2-ERK1/2 signaling pathway might be a novel pathway involved in HCC progression.

Key words: RASSF7; Hepatocellular carcinoma; Proliferation; Cell cycle; MEK1/2-ERK1/2 pathway.

Introduction

Worldwide, hepatocellular carcinoma (HCC) is one of the most common malignant cancers and the second cause of cancer-associated death (1,2). There are more than 750,000 people suffering from this deadly disease every year (3). The five-year overall survival rate remained poorly improved (4,5). Currently, surgery, chemotherapy, radiotherapy, and multimodality therapy is used to treat patients with HCC. Unfortunately, a satisfactory therapeutic effect has not been achieved because it is a highly complex disease. Hepatocellular carcinogenesis and progression are involving many genetic and environmental factors, and are multistep processes (6-10). Although the study of HCC has made great progress to date, the underlying molecular mechanisms are largely unclear yet. Therefore, elucidating the mechanisms underlying HCC progression is crucial for establishing new therapeutic targets for successful intervention.

The Ras-association domain family (RASSF) of proteins comprises 10 members from RASSF1 to RASSF10. One characteristic features of this family is the Ras-association domain (RA), which can be subdivided into C-terminally (RASSF1-6) or N-terminally (RASSF7-10). Members of the C-terminally RASSF proteins have been involved in many important biological processes, including cell cycle, survival, apoptosis, nuclear transport, microtubule stability and immune system regulation, and they are generally regarded as cancer suppressors (11,12). The N-terminal RASSF proteins represent a new cluster of potential Ras effectors and they may also have important biological functions, some of which could be distinct from previously studied Ras effectors. There is emerging evidence to indicate that two members of the N-terminal RASSF proteins may be cancer suppressors. The RASSF8 expression down-regulates and knocking down its expression promotes cell proliferation in lung cancer (13), the RASSF10 expression is epigenetically inactivated in thyroid cancers and leukaemias (14,15) and is down-regulated and acts as a novel tumor suppressor in hepatocarcinoma (16). In contrast, several studies have only demonstrated that RASSF7 expression is up-regulated in a range of cancers (17,18), but its function is still unknown in cancers.

In the present study, we analyzed RASSF7 expression in HCC tissues using data from the Cancer Genome Atlas (TCGA). We performed qRT-PCR in 82 pairs of HCC tissues and their normal tissues to detect RASSF7 expression in HCC. The function and molecular mechanism of RASSF7 in HCC were explored by a series of experiments. We found that RASSF7 expression was significantly up-regulated in HCC and the expression level was correlated with poor tumor histology, and
RASSF7 promoted HCC cell proliferation by activating MEK1/2-ERK1/2 signaling pathway. This is the first report describing some biological properties of RASSF7 protein in cancer. These findings may provide novel insight into the underlying mechanisms of HCC, and suggest that RASSF7 may serve as a potential molecular target for HCC treatments.

Materials and Methods

Tissues and cells

Tissues were obtained from 82 HCC patients treated at the Department of Hepatobiliary Surgery, the Affiliated Cancer Hospital of Zhengzhou University, PR China. The patients had not been pretreated with chemotherapy or radiotherapy prior to surgery. Informed consent was obtained from each patient. The study was approved by the Ethical Committee of the Affiliated Cancer Hospital of Zhengzhou University.

Normal human hepatic cell line (HL-7702) and HCC cell lines (Huh7, SMMC-7721 and HepG2) were obtained from the Cell Bank (Shanghai Genechem Co., Ltd., Shanghai, China). These cells were cultured in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco) at 37 °C in a humidified atmosphere containing 5% CO₂.

The construction of plasmid and transfection

Full-length human RASSF7 coding sequence was amplified and cloned into a pCMV2-GV146 vector. HepG2 cells were seeded in RPMI-1640 medium without antibiotics for 24 h. pCMV2-GV146-RASSF7 vector was transfected into HepG2 cells using Lipofectamine™.2000 (Invitrogen, Carlsbad, CA, USA). The cells were transfected and cultured for 48 h before performing assays.

siRNA synthesis and transfection

siRNA was pre-designed for RASSF7 gene silencing. Human RASSF7 siRNA (sense 5′-UAAUUGCU-GGCCAGCCUCCUGUA-3′, antisense 5′-UUUA-CAGGAGGCUGCAAGAUUA-3′) and negative siRNA (NC-siRNA, sense 5′-UUCUCCGAACGUUG-CACGUUAU-3′, antisense 5′-ACGUGACACGUUG-GAGAAUU-3′) were synthesized by GenePharma Corporation (SGC, Shanghai, China). Lipofectamine™.2000 was used to optimize siRNA transfection according to the manufacturer’s instructions. The siRNA complexes would be added to the cells and diluted to 80 nM in future experiment.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from HCC cells and tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. cDNA was synthesized according to the manufacturer’s protocol (Takara, Dalian, China). qRT-PCR was performed using the SYBR Green PCR kit (Takara, Dalian, China). β-Actin as a control for mRNAs. The primer sequences were as follows: RASSF7 forward: 5′-CAAGGG-CACGACTCTGCTTT-3′; RASSF7 reverse: 5′-GGCAT- CAGCGAAAATGACAGA-3′; β-Actin forward:5′-TGGCACCCAGCACAATGAA-3′; β-Actin reverse: 5′-CTAAGTCATAGTCGCTTGAAGCA-3′. All reactions were performed in triplicate with an IQ5 Multicolor qRT-PCR Detection System (Bio-Rad, USA).

Western blot analysis

HCC tissues, normal hepatic tissues and HCC cells were lysed in RIPA lysis buffer. Total protein was extracted and subjected to electrophoresis using 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was incubated with primary antibodies overnight and then incubated with secondary antibody for 2 hours at room temperature. The primary antibodies included rabbit monoclonal antibody (mAb) anti-RASSF7 (Abcam, USA), rabbit mAb anti-MEK1/2 (Santa Cruz, CA, USA), rabbit mAb anti-phospho-MEK1/2 (Santa Cruz, CA, USA), rabbit mAb anti-ERK1/2 (Santa Cruz, CA, USA), rabbit mAb anti-phospho-ERK1/2 (Santa Cruz, CA, USA), mouse mAb anti-Cyclin D1 (Santa Cruz, CA, USA), mouse mAb anti-β-Actin (Santa Cruz, CA, USA). The membranes were incubated with ECL (Amersham) for chemiluminescence detection. The luminescent signal was recorded and quantified with Syngene GBox (Syngene, UK).

Cell proliferation assay

MTT assay was used to evaluate the effect of RASSF7 on HepG2 cell viability. Cells (5,000 cells/well in 200 μl medium) were seeded into 96-well plates and incubated for 24 hours. These cells were treated with control vector (Ctrl), pCMV2-GV146-RASSF7 vector (RASSF7), NC-siRNA (80 nM), RASSF7 siRNA (80 nM) or RASSF7 vector + U0126 (MEK1/2 inhibitor U0126, 10 μM) (Sigma, USA) for 24, 48 and 72 hours, respectively. Then, 20 μl of 5 mg/ml MTT (Sigma, St Louis, MO, USA) solution was added per well and the cells were incubated for another 4 h. Cell viability was assessed at 492 nm using multi-microplate test system (POLARstar OPTIMA, BMG Labtechnologies, Germany). Each experiment was repeated at least three times. Data were presented as mean ± SD.

Cell counting assay

To measure cell proliferation, 2.5 × 10⁵ cells were plated in 60 mm diameter plates. HepG2 cells were treated with control vector, RASSF7 vector, NC-siRNA, RASSF7 siRNA or RASSF7 vector + U0126, respectively. The numbers of HepG2 cells were calculated at 24, 48 and 72 hours after treatment by using the Countess automated cell counter (Life Technologies Corp., Carlsbad, USA).

Cell cycle assay

HepG2 cells were cultured in 6-well plates for 24 hours and treated by control vector, RASSF7 vector, NC-siRNA, RASSF7 siRNA or RASSF7 vector + U0126 for 48 hours. The cells were fixed in 75 % ice cold ethanol at 4 °C for 12 hours. The fixed cells were stained with 50 μg/ml propidium iodide (PI) containing 50 μg/ml RNase A (DNase free) for 15 min at room temperature. Cell-cycle distributions were analyzed by fluorescence-activated cell sorting (FACS)Calibur, BD Biosciences, San Jose, CA, USA). There were three parallel wells in each independent experiment, and the procedures were carried out in triplicate.
RASSF7 promotes HCC cell proliferation.

Cell apoptosis assay

Cells were incubated in 6-well plates for 24 hours and treated with control vector, RASSF7 vector, NC-siRNA, RASSF7 siRNA or RASSF7 vector + U0126 for 48 hours. Then, the cells were stained with Annexin-VFITC Apoptosis Detection Kit (Invitrogen) according to the manufacturer’s instructions. Cells were detected by using a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA), and the apoptosis populations were analyzed by ModFit software.

Statistical analysis

Statistical analyses were performed using the SPSS version 21.0 statistical software. Student’s t test and one-way ANOVA were used according to the data characteristics. The data were presented as means ± Standard deviation (SD). Each experiment was performed at least 3 times independently. P < 0.05 were considered to be statistically significant.

Results

RASSF7 is significantly up-regulated in HCC tissues and is correlated with the clinicopathologic features of HCC

To explore the role of RASSF7 in HCC, we first analyzed the expression of RASSF7 in HCC tissues. TCGA data showed that RASSF7 expression was markedly higher in HCC tissues than in normal hepatic tissues (P < 0.01; Fig. 1A), the higher expression of RASSF7 was positively correlated with alpha-fetoprotein which has long been used as an effective biomarker for HCC detection (P < 0.01; Fig. 1B). In addition, the bioinformatics data also showed that RASSF7 expression was associated with poor tumor histology (P < 0.01; Fig. 1C). To verify the change of RASSF7 expression in HCC tissues, we examined its expression levels in 82 HCC tissues and matched adjacent non-tumor tissues by qRT-PCR. The results showed that RASSF7 mRNA expression was significantly up-regulated in HCC tissues compared with normal hepatic tissues (P < 0.01; Fig. 1D). The RASSF7 mRNA expression was associated with AFP, poor tumor histology and T stage (Table 1). This up-regulation was further verified through detecting three established GC cell lines, including Huh7, SMMC-7721 and HepG2. The results showed that RASSF7 mRNA levels in HCC cell lines were significantly higher than in normal human hepatic cell line HL-7702 (P < 0.01; Fig. 1E). The expression of RASSF7 protein was markedly higher in HCC tissues than in normal hepatic tissues, and was up-regulated in HCC cell lines (Fig. 1F and G). Taken together, RASSF7 is up-regulated in HCC cells,

Table 1. Patient characteristics and clinicopathologic correlation of RASSF7 expression.

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<th>P-value</th>
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*P < 0.05.
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suggested that RASSF7 may play a key role in the progression of HCC.

**Overexpression of RASSF7 promotes HCC cell proliferation and drives G1-S phase cell cycle transition by activating MEK1/2-ERK1/2 signaling pathway**

RASSF7 was expressed at high levels in HCC tissues, so we hypothesised that it might act as oncogene and that overexpression of RASSF7 could affect HCC progression. After RASSF7 overexpression vector had been transfected into HepG2 cells, we analyzed overexpression efficiency of RASSF7 vector in mRNA and protein levels. The results showed that RASSF7 mRNA and protein expressions significantly increased in RASSF7 overexpression vector group compared to control vector group (P < 0.01; Fig. 2A and B). MTT assay was performed to measure cell viability. We found that overexpression of RASSF7 significantly promoted HepG2 cell proliferation at 48 and 72 hours after transfection (P < 0.01; Fig. 2C). Cell counting assay also showed that overexpression of RASSF7 significantly promoted HepG2 cell proliferation (P < 0.01; Fig. 2D). The cell cycle is involved in the regulation of cell growth, so we measured the processes using a flow cytometer 48 hours after transfection. RASSF7 overexpression vector drove G1 to S phase cell cycle transition (P < 0.01; Fig. 2E). To examine the possible effects of RASSF7 overexpression vector on cell death, we observed the measurement of apoptosis by Annexin-V/PI staining. We found that RASSF7 overexpression vector inhibited apoptosis (P < 0.05; Fig. 2F). To further investigate the potential molecular mechanisms of RASSF7 in HCC, we measured the expressions of MEK1/2-ERK1/2 signaling pathway. No significant change was observed in the total protein expression of MEK1/2 and ERK1/2, but the phosphorylation levels of MEK1/2 and ERK1/2 significantly increased in RASSF7 overexpression group (Fig. 2G). Furthermore, we examined the expression of cell cycle regulator Cyclin D1. We found that RASSF7 overexpression vector promoted the expression of Cyclin D1 (Fig. 2G). These results indicate that RASSF7 could promote HCC HepG2 cell proliferation and drive cell cycle transition by activating MEK1/2-ERK1/2 signaling pathway.

**Silencing of RASSF7 suppresses HCC cell proliferation and induces G1-S arrest by inhibiting MEK1/2-ERK1/2 signaling pathway**

We specifically silenced RASSF7 expression in HepG2 cells by RASSF7 siRNA. After RASSF7 siRNA had been transfected into HepG2 cells, we analyzed knockdown efficiency of RASSF7 siRNA in mRNA and protein levels. The results showed that RASSF7 mRNA and protein expression decreased markedly in...
RASSF7 siRNA group compared to NC-siRNA group (P < 0.01; Fig. 3A and B). MTT assay showed that silencing of RASSF7 significantly suppressed cell proliferation at 48 and 72 hours after transfection (P < 0.01; Fig. 3C). Cell counting assay also showed that silencing of RASSF7 significantly inhibited HepG2 cell proliferation (P < 0.01; Fig. 3D). Cells were measured 48 hours after transfection, the cell cycles were arrested significantly at G1/G0 phase in RASSF7 siRNA group (P < 0.01; Fig. 3E). In addition, we also found that RASSF7 siRNA induced apoptosis (P < 0.01; Fig. 3F). The phosphorylation levels of MEK1/2 and ERK1/2 remarkably down-regulated in RASSF7 siRNA group, but there was no significant change in the total protein expression of MEK1/2 and ERK1/2 (Fig. 3G). RASSF7 siRNA also inhibited Cyclin D1 expression (Fig. 3G). The above data suggests that silencing of RASSF7 suppresses HCC cell proliferation and induces G1-S arrest by inhibiting MEK1/2-ERK1/2 signaling pathway.

Inhibiting of MEK1/2-ERK1/2 signaling eliminates the effects of RASSF7 overexpression on HCC cells

To demonstrate that RASSF7 exhibited oncogene function through MEK1/2-ERK1/2 signaling pathway, we inhibited MEK1/2-ERK1/2 signaling pathway with MEK1/2 inhibitor U0126 after RASSF7 overexpression. MTT assay showed that RASSF7 expression resulted in HCC cell proliferation after treatment with RASSF7 overexpression vector group (P < 0.01; Fig. 4A and B). Cell cycle assay showed that RASSF7 overexpression driven G1-S phase cell cycle transition, and RASSF7 + U0126 was able to arrest cell cycle (P < 0.01; Fig. 4C). RASSF7 + U0126 eliminated the effect of RASSF7 overexpression on apoptosis (P < 0.01; Fig. 4D). RASSF7 + U0126 also eliminated the effect of RASSF7 overexpression on p-ERK1/2 and Cyclin D1 expressions (Fig. 4E). These results further demonstrate that RASSF7 promotes HCC cell proliferation through activating MEK1/2-ERK1/2 signaling pathway.

Discussion

The aim of this study was to investigate the role of RASSF7 and to elucidate the molecular mechanisms of action in human HCC cells. Hepatic carcinogenesis is a multifactorial process involved in multiple genetic and epigenetic events. Dysregulation of oncogenes and anti-oncogenes plays a critical role in regulating cell survival, proliferation, apoptosis, cell cycle, invasion and migration in HCC (19-23). Up to now, it is only found that RASSF7 is up-regulated in several carcinomas, such as pancreatic tumors, ovarian carcinomas, endometrial cancer (17,18,24). RASSF7 was heavily methylated in neuroblastoma (25). It was found that oncogene MeCP2 could promote gastric cancer progression with regulating RASSF7 expression (26). These findings argue against RASSF7 having a tumor-suppressing role and...
raises the possibility that RASSF7 may even promote cancer formation. In this study, for the first time, TCGA data and our results all showed that RASSF7 expression was up-regulated in HCC tissues and associated with poor tumor histology. Our studies demonstrated that RASSF7 overexpression significantly promoted HCC cell proliferation and RASSF7 knockdown suppressed HCC cell growth, which indicates that RASSF7 might be a novel oncogene.

In this experiment, our results showed that RASSF7 expression drove G1-S phase cell cycle transition and silencing RASSF7 induced G1-S phase cell cycle arrest. The first gap phase (G1) of cell cycle is a critical stage when cells respond to environmental signals to determine cell fate such as proliferation, survival and cellular senescence. Cyclin D-CDK4/6 protein kinase complexes are important cell cycle regulators which govern the cellular progression through the G1-S phase of the cell cycle (27,28). In a cell cycle-independent manner, the expressions of CDK4/6 are stable, but Cyclins (D1, D2 and D3) expressions fluctuate during the cell cycle (29,30). This suggests that the roles of different Cyclin D are critical in controlling cell cycle progression. Cyclin D1 is an important regulator which governs the cellular progression through the G1-S phase of the cell cycle. Cyclin D1 leads to release of the E2F transcription factors and drives cell entry into the S phase of the cell cycle after the extracellular mitogenic stimulation. Previous studies found that Cyclin D1 is involved in human tumorigenesis and cancer progression, such as prostate cancer, breast cancer and hepatocellular carcinoma (31-33). Our results showed that RASSF7 overexpression promoted the expression of Cyclin D1 and silencing RASSF7 inhibited Cyclin D1 expression. These results suggest that RASSF7 might drive cell entry into the S phase of cell cycle by promoting Cyclin D1 expressions.

Ras/MEK/ERK pathway is one of most important signaling pathways, which promote cell survival, proliferation, metastasis, death, angiogenesis as well as other important cellular responses in human cancers (34-37). Ras/MEK/ERK pathway represents key signals. EBioMedicine 2016; 12:55-67.


