RESEARCH ARTICLE

Lentivirus-mediated Silencing of Rhomboid Domain Containing 1 Suppresses Tumor Growth and Induces Apoptosis in Hepatoma HepG2 Cells

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Abstract

Rhomboids were identified as the first intramembrane serine proteases about 10 years ago. Since then, the study of the rhomboid protease family has blossomed. Rhomboid domain containing 1 (RHBDD1), highly-expressed in human testis, contains a rhomboid domain with unknown function. In the present study, we tested the hypothesis that RHBDD1 was associated with proliferation and apoptosis in hepatocellular carcinoma using recombinant lentivirus-mediated silencing of RHBDD1 in HepG2 cells. Our results showed that down-regulation of RHBDD1 mRNA levels markedly suppressed proliferation and colony formation capacity of HepG2 human hepatoma cancer cells in vitro, and induced cell cycle arrest. We also found that RHBDD1 silencing could obviously trigger HepG2 cell apoptosis. In summary, it was demonstrated that RHBDD1 might be a positive regulator for proliferative and apoptotic characteristics of hepatocellular carcinoma.

Keywords: RHBDD1 - hepatocellular carcinoma - lentivirus - siRNA

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Introduction

Many functionally important membrane proteins are cleaved within their transmembrane helices to become activated (Ha, 2009). This process is catalyzed by a class of highly specialized and membrane-bound proteases, named as intramembrane proteases, including metalloproteases, aspartyl proteases and the rhomboid-like family (De Strooper et al., 1998; Akiyama et al., 2004; Lemberg and Freeman, 2007). Although initially seen as purely degradation enzymes, it has become clear that they are involved in several crucial cellular and developmental processes, such as, cell signaling, apoptosis and blood clotting (Brown et al., 2000; Kroos and Yu, 2000; Weihofen and Martoglio, 2003; Wolfe and Kopan, 2004; Freeman, 2008). In the past years, a growing number of papers have been published to address the structural features, functions and mechanisms of these membrane proteins.

Drosophila rhomboid-1, originally discovered as a key genetic factor in the fly epidermal growth factor receptor (EGFR) signaling pathway, is the founding member of the family (Urban et al., 2001). Subsequent researches showed that rhomboid proteases are present in most organisms. In yeast, there are two rhomboids, named Rbd1p and Rbd2p. Rbd1p is localized in the inner mitochondrial membrane and mutant cells have disrupted mitochondria membrane remodeling (McQuibban et al., 2003). In parasites, a rhomboid-like protease plays an important role in host cell invasion (Howell et al., 2005). Despite the functions in lower eukaryotes, the functions of vertebrate rhomboid-like family members remain unclear. However, several investigations have provide strong evidence that rhomboid protease may play an important role in cancer cell growth and apoptosis. For example, human rhomboid family-1 (RHBDF1), a novel family member of rhomboid proteases, participates in the modulation of GPCR-mediated EGFR transactivation and disrupts growth signals in several cancer cell growth, including epithelial cancer, breast cancer, and head and neck squamous cancer cells (Yan et al., 2008; Zou et al., 2009). Rhomboid domain containing 1 (RHBDD1), an important gene highly expressed in the testis, is shown to participate in the cleavage of BIK and modulate BIK-mediated apoptotosis (Wang et al., 2008). RHBDD2, markedly over-expressed in primary tumors from patients with recurrent disease, has been shown to regulate cell proliferation of breast cancer cells (Abba et al., 2009).

Consequently, to determine the potential role of RHBDD1 in human primary hepatocellular carcinoma (HCC), we studied the effect of RHBDD1 knockdown in HCC HepG2 cells via a lentivirus-mediated silencing system. Our data show that in vitro RHBDD1 silencing regulates HepG2 cell proliferation and apoptosis.

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Materials and Methods

Lentivirus construction

The lentivirus-mediated silencing system targeting RHBDD1 purchased from Shanghai Genechem contains three vectors: pGCSIL-GFP vector and two packing vectors, pHelper 1.0 and pHelper 2.0. Briefly, small interfering (5'-GCTGGGAATCTTTGGACTA-3') sequence targeting RHBDD1 and scrambled control RNA sequence (5'-TTTCCGAAACGTGTCACGT-3') were designed. Then the stem-loop short hairpin RNA (shRNA) sequences based on RHBDD1-siRNA and control-siRNA were subcoloned into pGCSIL-GFP vectors, named as RHBDD1-shRNA and control-shRNA. To generate reconstructed lentiviruses, the pGCSIL-GFP vectors were cotransfected with pHelper 1.0 and 2.0 vectors into 293T cells using a LipofectamineTM 2000 kit (Invitrogen) according to the manufacturer’s instructions. Reconstructed lentiviruses were harvested by centrifugation after 72 hrs.

Cell culture and transfection

The human hepatoma cancer cell line HepG2 was purchased from the American Type Culture Collection (ATCC). The cells were grown in RPMI-1640 culture medium (Invitrogen, Carlsbad, CA) containing 1% penicillin/streptomycin, at 37°C with 5% CO₂. In the following experiments, purified RHBDD1-shRNA and control-shRNA lentiviruses were applied to HepG2 cells. To monitor the infection efficiency, infected cells were collected and subjected to RNA extraction and reverse transcription. Total RNA was transcripted into cDNA with random primers. QRT-PCR was performed according to the ABI manufacturer’s protocols. Relative expression levels of RHBDD1 (Applied Biosystems, Bedford, MA, USA) were detected. Two pairs of primers were used in this experiment. GAPDH-F, 5'-TGACTTACACCCGCAACCA-3', GAPDH-R, 5'-CACCCTGTGCTAGCCAAA-3'; RHBDD1-F, 5'-GCAGGACTGAGTGAAGAAGAAC-3', RHBDD1-R, 5'-GTGAGAGATGAAACCCGTAGG-3'. All samples were examined in triplicate.

RNA extraction, reverse transcription and qRT-PCR

Infected (RHBDD1-shRNA and control-shRNA) HepG2 cells were collected and subjected to RNA extraction and reverse transcription. Total RNA was isolated by Trizol (Invitrogen, Carlsbad) reagent, and 2 μg total RNA was transcribed into cDNA with random primers. QRT-PCR was performed according to the ABI (Applied Biosystems, Bedford, MA, USA) manufacturer’s protocols. Relative expression levels of RHBDD1 mRNA were compared to the levels of reference gene (GAPDH) by comparative cycle threshold (ct) method as fold difference = 2^–(ΔΔct of target gene–ΔΔct of reference). Two pairs of primers were used in this experiment. GAPDH-F, 5'-TTCTCGGAACGTGTCACGT-3', GAPDH-R, 5'-TTTCCGAAACGTGTCACGT-3'. RHBDD1-F, 5'-GCAGGACTGAGTGAAGAAGAAC-3', RHBDD1-R, 5'-GTGAGAGATGAAACCCGTAGG-3'. All samples were examined in triplicate.

Cell cycle analysis by flow cytometry

Cells were analyzed by FACS Calibur (BD Biosciences, USA). The cell cycle distribution in G1, S or G2/M phases in both groups were determined. All samples were examined in triplicate.

Cell cycle analysis by flow cytometry

Flow cytometry was used to analyze the percentage of cells in each phase. In brief, HepG2 cells were synchronized by serum starvation for 24 hrs. Then cells were transfected with RHBDD1-shRNA or control-shRNA lentiviruses. After 48 hrs incubation, cells were trypsinized, washed with PBS and fixed in 70% ethanol. The fixed cells were then incubated in propidium iodide (PI, 100 μg/mL) (Sigma, USA)/PBS solution with Rnase (10 μg/mL) for at least 30 min at 37 °C in dark. PI stained cells were analyzed by a FACs caliber II sorter and Cell Quest FACS system (BD Biosciences, USA). The cell cycle distribution in G1, S or G2/M phases in both groups was determined. All samples were examined in triplicate.

Apoptosis analysis by Annexin-V

The apoptotic effect of RHBDD1 in HepG2 cells was examined by flow cytometry using Annexin V Apoptosis Detection Kit (eBioscience, San Diego, CA, USA). Firstly, HepG2 cells were infected with lentiviruses containing RHBDD1-shRNA or control-shRNA. After 72 hrs, infected cells were trypsinized, washed with D-Hanks buffer, PBS buffer and 1× binding buffer, consecutively. And cells were subjected to 1× staining buffer containing annexin V-APC for 15 min. The percentage of apoptotic cells was analyzed by FACs Calibur (BD Biosciences, USA).

Statistical analysis

The data shown are the means ± SD of three experiments. Statistical significance was estimated with Student’s t-test. A P-value of less than 0.05 was considered significant in different experiments.

Results

The lentiviruses containing RHBDD1-shRNA or control-shRNA were co-transfected into HepG2 cells. After 48 hrs, these cells were examined via fluorescence microscope. As shown in Figure 1A, The GFP tag in lentiviruses enables visualization of transfection efficiency via bright (Figure 1A, left) and fluorescence (Figure
A. For both group, HepG2 cells infected with lentiviruses were taken 48 hrs after lentivirus treatment at a magnification of x200. B. Identification of RHBD1 knockdown efficiency using shRNA lentivirus via qRT-PCR. GAPDH was used as reference gene. *P<0.05 compared to control.

Figure 2. RHBD1-shRNA Suppressed HepG2 Cell Growth. A. For both group, HepG2 cells infected with lentiviruses (RHBD1-shRNA or control-shRNA) were re-cultured in 96-well plates. The plates were read by Cellomics ArrayScan (Thermo) in the next 5 days, and the cell numbers were expressed as fold change. In each time point, the cell number of RHBD1-shRNA treated cells was compared to that of control-shRNA. B. The number of colonies HepG2 cells formed in both groups. Cells were stained with Giemsa and observed by routine bright-field microscopy, and colonies (>50 cells) were counted. **P<0.01 compared to control.

Reduction of RHBD1 caused growth delay of human HepG2 cells

To assess the role of RHBD1 in the survival of human HepG2 cells, cell proliferation assays were performed to examine the growth of HepG2 cells with reduced RHBD1 mRNA expression. As shown in Figure 2A, down-regulation of RHBD1 resulted in significant delay of HepG2 cell growth as compared to the control-shRNA.

Suppression of RHBD1 induced G2/M phase arrest

In order to find out whether RHBD1-shRNA delays HepG2 cell proliferation partly through regulation of cell cycle, we then examined cell cycle distribution by flow cytometry. Firstly, cells were infected with lentiviruses containing RHBD1-shRNA or control-shRNA. After 48 hrs incubation, cells were harvested and analyzed by PI staining and flow cytometry. Compared with control-shRNA treated HepG2 cells, RHBD1-shRNA treated cells showed a substantial decrease in G1-phase (P<0.001) and an increase in G2/M phase populations (P<0.001) in HepG2 cells (Figure 3).

Decreased RHBD1 induced apoptosis

To determine the apoptotic effect of RHBD1-shRNA on HepG2 cells, an Annexin V apoptosis assay was performed. As Figure 4 shows, more than 95% of the cells showed an increase in the number of Annexin V positive cells in RHBD1-shRNA treated group compared to control-shRNA treated group.
Silencing of RHBDD1 Suppresses Hepatoma Proliferation

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It is still unclear whether rhomboid family is involved in proliferation and invasion (Zou et al., 2009). However, of the biological characteristics of cancers, including chemotherapy, can inhibit the proliferation of HCC cells

for HCC (Desbois-Mouthon et al., 2006; Berasain et al., 2012; Gao et al., 2012). Moreover, various sites of EGF receptor and related kinases can be targets of treatments for HCC (Desbois-Mouthon et al., 2006; Berasain et al., 2012; Sugawa et al., 2012). For example, Cetuximab is a chimeric monoclonal Ig G1 antibody directed against the EGFR and blocks binding of endogenous EGFR ligands (Furuse, 2008). Cetuximab alone or in combination of erlotinib, other tyrosine-kinase inhibitors and cytotoxic chemotherapy, can inhibit the proliferation of HCC cells in vivo and in vitro (Sanoff et al., 2011; Chen et al., 2012; Ezzoukhry et al., 2012).

In Drosophila, rhomboid intramembrane proteases catalyse the release of EGF-family ligands. Intriguingly, in human, members of rhomboid intramembrane proteases have also been shown to play an important role in the activation of EGFR (Zou et al., 2009; Adrain et al., 2011). Moreover importantly, the disruption of EGFR signaling by rhomboid family may lead to the alterations of the biological characteristics of cancers, including proliferation and invasion (Zou et al., 2009). However, it is still unclear whether rhomboid family is involved in HCC development. Therefore, in the present study, we try to identify the biological functions of RHBDD1, a new member of the Rhomboid family, in the proliferation and apoptosis of HepG2 cells via a lentivirus-mediated silencing system (Figure 1).

We found that lentivirus-mediated down-regulation of RHBDD1 in human hepatoma cancer cell line HepG2 cells leads to marked inhibition of cell proliferation (Figure 2). Moreover, the suppression of HepG2 cell growth may partly resulted from the dysregulation of cell cycle progressing. As Figure 3 shows, RHBDD1-shRNA induced an accumulation of HepG2 cells in G2/M phase (23.1%), as compared to control-shRNA (11.3%). We thus suspect that the inhibition of HepG2 cell growth and cell cycle delay may be triggered by EGFR related signaling pathway.

Knowing that the RHBDD1-involved proteolytic modification is upstream of the BIK protein degradation pathway and knock-down of RHBDD1 can enhance BIK-mediated apoptosis, we also evaluate the apoptotic effect of RHBDD1-shRNA in HepG2 cells. Our data shows that inhibition of RHBDD1 was effective in apoptosis induction in HepG2 cells, causing an increase of the number of apoptotic cells from 2.8% in the control groups to 21.7% in the RHBDD1-shRNA group. It is conceivable that the RHBDD1 gene regulate HepG2 cell apoptosis through BIK-mediated pathway. However, the underlying mechanisms responsible for the biological function of RHBDD1 need further investigations. The EGFR is a member of a family of four closely related receptors: EGFR (ErbB-1), HER-2/Neu (ErbB-2), HER-3 (ErbB-3) and HER-4 (ErbB-4). Interestinglyly, the expression of HER may play a role in the invasion, metastasis and progression of HCC (Bacaktsiz et al., 2008; Zhang et al., 2010; Xu et al., 2011). Therefore, whether RHBDD1 participates in the above signaling pathways during HCC development remains wide open.

In conclusion, our findings strongly suggest that the RHBDD1 gene plays a critical role in the regulation of HCC cell growth and apoptosis, indicating that RHBDD1 may serve as a potential drug target for hepatoma cancer therapeutics.

**References**


intramembrane proteolysis: a control mechanism conserved from bacteria to humans. Cell, 100, 391-8.