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-medicated apoptosis (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'- GCTGGGATTCTTGTTGGACTA-3') sequence targeting RHBDD1 and scrambled control RNA sequence (5'- TTCTCCGAACGTGTCACGT-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 re-constructed 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. Re-constructed 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% CO_2. 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 for fluorescence microscopy and quantitative real-time PCR (qRT-PCR) assay 48 hrs after lentivirus treatment.

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 transcripted 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 = $2^{-(\triangle ct \text{ of target gene} - \triangle ct \text{ of reference})}$. Two pairs of primers were used in this experiment. GAPDH-F, 5'- TGACTTCAACAGCGACACCCA-3', GAPDH-R, 5'-CACCCTGTTGCTGTAGCCAAA-3'; RHBDD1-F, 5'-GCAGGACTGAGTGAAGAAGAAC-3', RHBDD1-R, 5'-GTGAGAGATGAAACCCGTAGG-3'. All samples were examined in triplicate.

Cell proliferation assay

Cell proliferation assay was determined by measuring GFP-positive cells using the Cellomics Array Scan VTI HCS (Thermo Fisher Scientific, Pittsburgh, PA). Briefly, HepG2 cells were infected with lentiviruses (RHBDD1-shRNA or control-shRNA) for 48 hrs, and infected cells were re-cultured in 96-well plates with a density of 2000 cells/well. Plates were read on the Thermo Scientific Cellomics ArrayScanat indicated time points for cell count. The cell count was expressed as fold changes.

Colony formation assay

HepG2 cells were cultured in 24-well plates and treated with RHBDD1-shRNA or control-shRNA lentiviruses. And 0.5 mL under layers consisting of 0.8% agar medium was prepared in 6-well plates. After 48 hrs of incubation, cells were washed, re-cultured in the prepared 6-well plates, and allowed to form natural colonies. After 14 days, cells in both groups were subjected to Giemsa staining. Firstly, cells were washed and fixed by paraformaldehyde. Then fixed cells were washed twice with PBS solution, treated with Giemsa (MBCHEM, New Jersey, USA) for 10 min, washed 3 times by ddH_2O, and then photographed with a digital camera. The number of colonies (>50 cells/colony) were counted.

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

Suppression of RHBDD1 by siRNA in human HepG2 cells

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

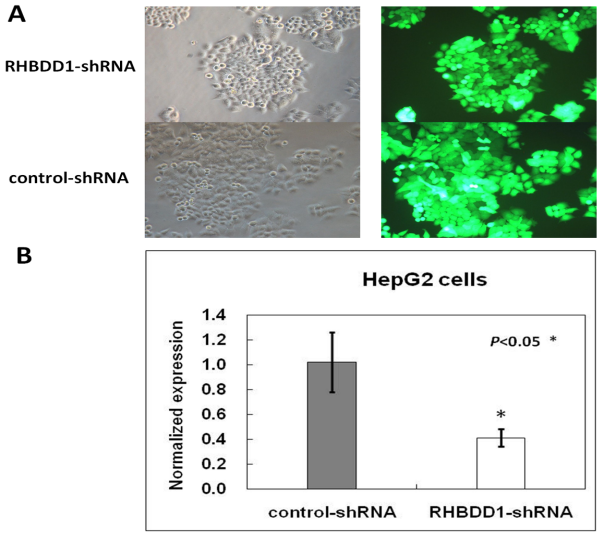

Figure 1. Lentivirus-mediated Silencing of RHBDD1. A. Lentivirus infection in human hepatoma HepG2 cells. Fluorescence photomicrographs of GFP-positive cells infected by lentivirus. Pictures were taken 48 hrs after lentivirus treatment at a magnification of ×200. B. Identification of RHBDD1 knockdown efficiency using shRNA lentivirus via qRT-PCR. GAPDH was used as reference gene. *P<0.05 compared to control

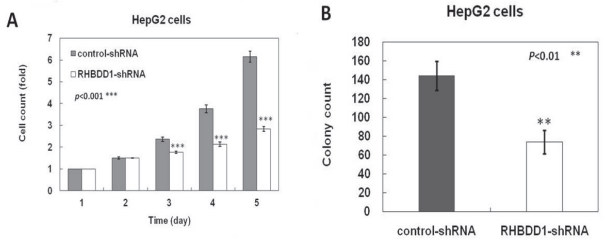

Figure 2. RHBDD1-shRNA Suppressed HepG2 Cell Growth. A. For both group, HepG2 cells infected with lentiviruses (RHBDD1-shRNA or control-shRNA) were re-cultured in 96-well plates. The plates were read by Cellomics ArrayScan (Thermo) in the next 5 days, and the cell numbers were expressed as fold change. In each time point, the cell number of RHBDD1-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

1A, right) microscope. In both groups, >80% cells were successfully infected. To confirm RHBDD1-shRNA silencing effect, cells were lysated, followed by RNA extraction, reverse transcription and qRT-PCR. Over 60% RHBDD1 mRNA was decreased after treatment using RHBDD1-shRNA as compared to that using control-shRNA (Figure 1B). Therefore, the knockdown efficiency of RHBDD1 by lentiviral system was verified.

Reduction of RHBDD1 caused growth delay of human HepG2 cells

To assess the role of RHBDD1 in the survival of human HepG2 cells, cell proliferation assays were performed to examine the growth of HepG2 cells with reduced RHBDD1 mRNA expression. As shown in Figure 2A, down-regulation of RHBDD1 resulted in significant delay of HepG2 cell growth as compared to the control-shRNA

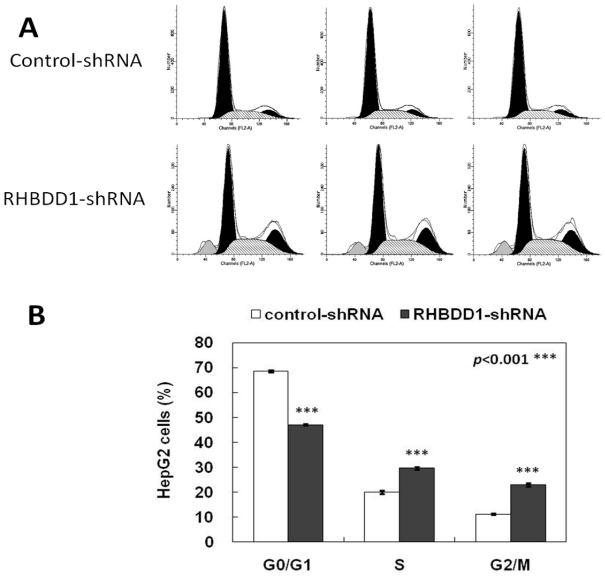

Figure 3. RHBDD1-shRNA Caused A G2/M Arrest. After serum deprivation, cells were incubated with RHBDD1-shRNA or control-shRNA containing lentiviruses for 48 hrs. The nuclei were stained with propidium iodide and the cell cycle was analyzed via flow cytometry as described in materials and methods. A. Flow cytometry histograms of HepG2 cells following lentivirus infection in three parallel experiments. B. Analysis of the subpopulations of cells in cell cycle phases G1, S, and G2/M. ***P<0.001 compared to control-shRNA

on the third day following lentivirus infection (P<0.001). On the fourth and fifth day, the growth inhibitory effect of RHBDD1-shRNA was more remarkable.

To study the role of RHBDD1 in human HepG2 cell tumorigenesis in vitro, we further evaluated the colony formation capacity of HepG2 cells in soft agar. As Figure 2B shows, reduction of RHBDD1 in HepG2 cells caused a substantial reduction in colony formation in soft agar as compared with the control group (P<0.01). The RHBDD1-shRNA treated group formed 74.0 ± 12.5 colonies; while control-shRNA formed 144.3 ± 15.6 colonies in 14 days after lentivirus treatment. Thus, the reduced RHBDD1 expression ultimately caused significant inhibition of growth in human HepG2 cells, suggesting that RHBDD1 is critical for tumorigenicity of human HepG2 cells in vitro.

Suppression of RHBDD1 induced G2/M phase arrest

In order to find out whether RHBDD1-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 RHBDD1-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, RHBDD1-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 RHBDD1 induced apoptosis

To determine the apoptotic effect of RHBDD1-shRNA on HepG2 cells, an Annexin V apoptosis assay was performed. As Figure 4 shows, more than 95% of the

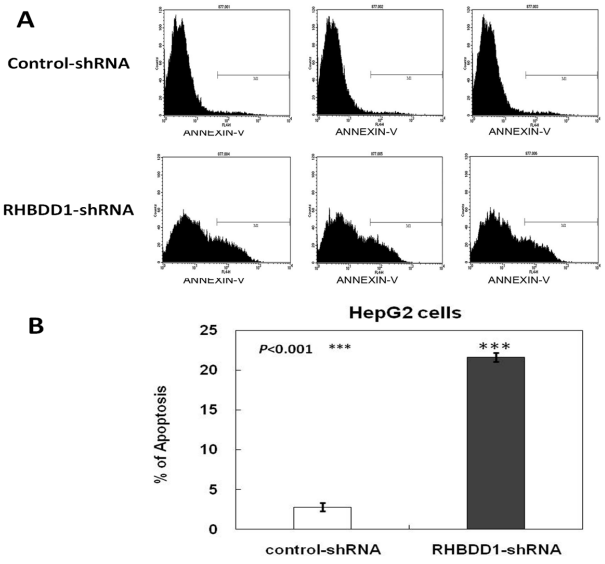

Figure 4. RHBDD1-shRNA Induced HepG2 Cell Apoptosis. A. Cytogram of Annexin V binding in HepG2 cells treated with lentiviruses containing RHBDD1-shRNA or control-shRNA. B. Analysis of apoptotic cells in both group. ***P<0.001 compared to control-shRNA

control-shRNA cells were viable, while less than 80% of the RHBDD1-shRNA cells were alive. The apoptotic cells in RHBDD1 inhibition group was 7.7 times that in control group (P<0.001). Our data indicated that RHBDD1 has a potential role in the regulation of apoptosis in human HepG2 cells.

Discussion

HCC is one of the most common malignancies worldwide and is estimated to cause half a million deaths annually (Zeng et al., 2012). Accumulating researches have reported that EGFR and key members in EGFR signaling are aberrantly expressed in HCC, contributing to invasion, metastasis and resistance to chemotherapy and radiotherapy (Yoneda et al., 2011; Ezzoukhry 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; Sogawa 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). More 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). Interestingly, the expression of HER may play a role in the invasion, metastasis and progression of HCC (Bacaksiz 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

Abba MC, Lacunza E, Nunez MI, et al (2009). Rhomboid domain containing 2 (RHBDD2): a novel cancer-related gene over-expressed in breast cancer. *Biochim Biophys Acta*, **1792**, 988-97.

Adrain C, Strisovsky K, Zettl M, et al (2011). Mammalian EGF receptor activation by the rhomboid protease RHBDL2. *EMBO Rep*, **12**, 421-7.

Akiyama Y, Kanehara K, Ito K (2004). RseP (YaeL), an Escherichia coli RIP protease, cleaves transmembrane sequences. *Embo J*, **23**, 4434-42.

Bacaksiz A, Sahin FI, Bilezikci B, et al (2008). Determination of HER-2/Neu status in hepatocellular carcinoma cases. *Genet Test*, **12**, 211-4.

Berasain C, Nicou A, Garcia-Irigoyen O, et al (2012). Epidermal growth factor receptor signaling in hepatocellular carcinoma: inflammatory activation and a new intracellular regulatory mechanism. *Dig Dis*, **30**, 524-31.

Brown MS, Ye J, Rawson RB, et al (2000). Regulated

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

Chen W, Shen X, Xia X, et al (2012). NSC 74859-mediated inhibition of STAT3 enhances the anti-proliferative activity of cetuximab in hepatocellular carcinoma. *Liver Int*, **32**, 70-7.

De Strooper B, Saftig P, Craessaerts K, et al (1998). Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature*, **391**, 387-90.

Desbois-Mouthon C, Cacheux W, Blivet-Van Eggelpoel MJ, et al (2006). Impact of IGF-1R/EGFR cross-talks on hepatoma cell sensitivity to gefitinib. *Int J Cancer*, **119**, 2557-66.

Ezzoukhry Z, Louandre C, Trecherel E, et al (2012). EGFR activation is a potential determinant of primary resistance of hepatocellular carcinoma cells to sorafenib. *Int J Cancer*, **131**, 2961-9.

Freeman M (2008). Rhomboid proteases and their biological functions. *Annu Rev Genet*, **42**, 191-210.

Furuse J (2008). Growth factors as therapeutic targets in HCC. *Crit Rev Oncol Hematol*, **67**, 8-15.

Gao J, Yu Y, Zhang Y, et al (2012). EGFR-specific PEGylated immunoliposomes for active siRNA delivery in hepatocellular carcinoma. *Biomaterials*, **33**, 270-82.

Ha Y (2009). Structure and mechanism of intramembrane protease. *Semin Cell Dev Biol*, **20**, 240-50.

Howell SA, Hackett F, Jongco AM, et al (2005). Distinct mechanisms govern proteolytic shedding of a key invasion protein in apicomplexan pathogens. *Mol Microbiol*, **57**, 1342-56.

Kroos L, Yu YT (2000). Regulation of sigma factor activity during Bacillus subtilis development. *Curr Opin Microbiol*, **3**, 553-60.

Lemberg MK, Freeman M (2007). Functional and evolutionary implications of enhanced genomic analysis of rhomboid intramembrane proteases. *Genome Res*, **17**, 1634-46.

McQuibban GA, Saurya S, Freeman M (2003). Mitochondrial membrane remodelling regulated by a conserved rhomboid protease. *Nature*, **423**, 537-41.

Sanoff HK, Bernard S, Goldberg RM, et al (2011). Phase II Study of Capecitabine, Oxaliplatin, and Cetuximab for Advanced Hepatocellular Carcinoma. *Gastrointest Cancer Res,* **4**, 78-83.

Sogawa C, Tsuji AB, Yoshida C, et al (2012). Novel human monoclonal antibody against epidermal growth factor receptor as an imaging probe for hepatocellular carcinoma. *Nucl Med Commun*, **33**, 719-25.

Urban S, Lee JR, Freeman M (2001). Drosophila rhomboid-1 defines a family of putative intramembrane serine proteases. *Cell*, **107**, 173-82.

Wang Y, Guan X, Fok KL, et al (2008). A novel member of the Rhomboid family, RHBDD1, regulates BIK-mediated apoptosis. *Cell Mol Life Sci* **65**, 3822-9.

Weihofen A, Martoglio B (2003). Intramembrane-cleaving proteases: controlled liberation of proteins and bioactive peptides. *Trends Cell Biol*, **13**, 71-8.

Wolfe MS, Kopan R (2004). Intramembrane proteolysis: theme and variations. *Science*, **305**, 1119-23.

Xu W, Cao L, Chen L, et al (2011). Isolation of circulating tumor cells in patients with hepatocellular carcinoma using a novel cell separation strategy. *Clin Cancer Res*, **17**, 3783-93.

Yan Z, Zou H, Tian F, et al (2008). Human rhomboid family-1 gene silencing causes apoptosis or autophagy to epithelial cancer cells and inhibits xenograft tumor growth. *Mol Cancer Ther,* **7**, 1355-64.

Yoneda N, Sato Y, Kitao A, et al (2011). Epidermal growth factor induces cytokeratin 19 expression accompanied by increased growth abilities in human hepatocellular carcinoma. *Lab Invest,* **91**, 262-72.

Zeng QA, Qiu JL, Zou RH, et al (2012). Clinical features and outcome of multiple primary malignancies involving hepatocellular carcinoma: A long-term follow-up study. *BMC Cancer,* **12**.

Zhang JK, Pan PL, Wu YM, et al (2010). [Expression of HER-2/neu oncogene in hepatocellular carcinoma and the clinical implications]. *Nan Fang Yi Ke Da Xue Xue Bao,* **30**, 326-8.

Zou H, Thomas SM, Yan ZW, et al (2009). Human rhomboid family-1 gene RHBDF1 participates in GPCR-mediated transactivation of EGFR growth signals in head and neck squamous cancer cells. *Faseb J,* **23**, 425-32.