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Overexpression of HSD17B13 induces G1 arrest in hepatic cell line detected by anti-HSD17B13 recombinant rabbit monoclonal antibody

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Abstract

Hepatocellular carcinoma (HCC) is one of the most prevalent tumors worldwide. Hydroxysteroid 17-beta dehydrogenase 13 (HSD17B13), which is newly identified as a liver lipid droplet associated protein, is recently found to be down-regulated in HCC. However, the effects of HSD17B13 on the underlying mechanisms of HCC have not yet been fully explored. For this purpose, HSD17B13 gene is knockdown with CRISPER/Cas9 technique in HCC cell line. HSD17B13 expression is detected by Flow cytometry and Western blotting with recombinant rabbit monoclonal antibodies (clone OTIR3G2) against HSD17B13 which are screened for use in immunohistochemistry, western blot, and ELISA application. Overexpressed HSD17B13 induces hepatic cell line G1 capture and effects several cell cycle related protein expression, including p21, p27, MMP2 and MMP3. Then the proliferation and cell cycle are suppressed. HSD17B13 gene knockdown will reverse the suppression. These data indicates that HSD17B13 play important roles in the progression of HCC lesions. HSD17B13 might be a potential immune-oncology marker for HCC.

Introduction

HCC has been proved that the major cause is Non-alcoholic fatty liver disease (NAFLD) in many countries. HSD17B13 is identified to be a liver enriched, hepatocyte-specific, lipid droplet associate protein. It has been reported to be strongly associated with the development and progression of NAFLD in both human and mice. It has been reported HSD17B13 protein expression were significantly down regulated in HCC tissues. And the *in vitro* study showed HSD17B13 induced an accumulation of cells in G1 phase and reduction of cells in S and G2 phases via up regulating the expression of P21, P27 and MMP2. But the current studies focused on the pathogenic study and could not claim the HSD17B17 protein expression with a confirmed quantity because of the shortness of monoclonal antibody. In our study, we use Crisper/Cas9 technique knockdown HSD17B13 in SK-HEP-1 cells which is one of the HCC cell lines. HSD17B13 protein expression was detected by OriGene rabbit monoclonal antibody anti HSD17B13, TA592045, cloneOTI3G2. Our aim is investigating HSD17B13 expression and its role in hepatocellular carcinoma cells.

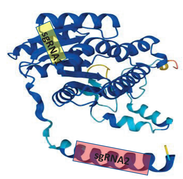
Design & Methods

Antibody Development

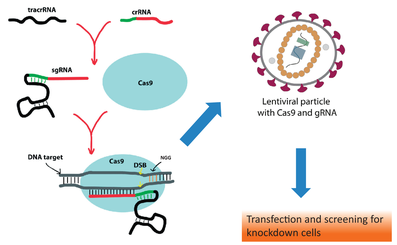
Rabbit recombinant monoclonal antibody was developed using B cells from peripheral blood. Briefly, B cells were isolated from the whole blood of immunized rabbit with HSD17B13 peptides. Cells from immune response positive wells were selected and identified. Rabbit IgG light chain and heavy chain variant were amplified and cloned into expression vectors. Positive antibody clones were sequenced to ensure to be produced correctly. Antibody were purified after both light and heavy chain were co-transfected into 293-6E cells. More than 10 positive HSD17B13 clones were screened by immunocytochemistry and immunohistochemistry. We choose clone OTI3G2 for this study.

Crisper/Cas9 knockdown

To generate HSD17B13 gene knockout cells, SK-HEP1 cells were infected with lentiviruses carrying a *HSD17B13*-targeting sgRNA (sgRNA1: 5'-AGTGGGTGATGTAACAATCG-3';sgRNA2:5'-CTTCACCAACGACTCCAAGT-3'). The infected cells were selected with puromycin (2 μg/ml) for stable *HSD17B13* sgRNA expression. Single clones were propagated and picked. Western blot was used to verify the efficiency of knockout in cells (anti HSD17B13 rabbit monoclonal antibody; clone OTI3G2).



HSD17b13 protein 3D structure
Yellow area shows sgRNA1 primer's location. Pink area shows sgRNA2 primer's location



Lentiviral particle with Cas9 and gRNA

Transfection and screening for knockdown cells

WB, ELISA and flow cytometry

Total cell lysates were solubilized in RIPA buffer and the protein content of each lysate was checked by bicinchoninic acid assay (BCA). Equal volumes of each sample were loaded onto SDS-PAGE. The gel was then transferred to nitrocellulose membrane and incubated with primary antibody (OTI3G2) for 1 hr at room temperature and then with secondary antibody for about 1 hr. The visualization of the proteins was performed by enhanced chemiluminescence reagent.

Cell lysates were adjusted to the same concentration and equal amount was coated on high binding ELISA plates at 4C overnight. Then incubated with primary antibody for 1 hr at room temperature and secondary antibody for another 1 hr. OD was read at 450 for AP-PNPP reaction.

Cell cycle analysis to check whether HSD17B13 triggers cell cycle arrest. The knockout cells were cultured for 24 hrs, trypsinized and fixed with 70% ethanol, then staining with PI for 30 min. Finally, the cells were subjected to flow cytometry.

Transfection

Adjust cells to approximately 80% confluent at time of transfection. A total of 5 μg of HSD17B13 DNA was diluted to 50 μl with transfection media. 15ul of polyethylenimine (PEI) transfection reagent was diluted in transfection media and added to the plasmid mixture. The DNA: PEI combination was incubated at room temperature for 30 min after gently mixing. A total of 100 μl of the mixture was added to each plate. Cells were maintained at 37°C and 5% CO_2 for 72 h.

Results

Fig 1. Key Steps To Recombinant Rabbit Monoclonal Antibody Development

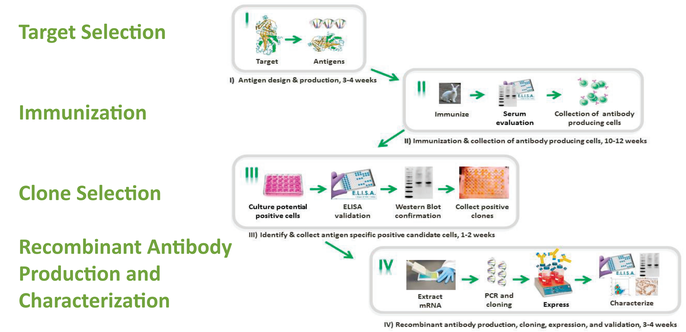


Figure 1. Cartoon of protocol for recombinant antibody development of rabbit monoclonal antibodies. There are four major production steps each area requires multiple steps of analysis to allow for selection of highest specificity antibodies.

Fig 2a HSD17B13 knockdown clones confirmed by WB

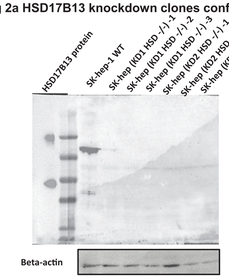


Figure 2a: Analysis knockdown clones for HSD17B13 by Western blot all the lysates were loaded as same amount. Primary antibody is anti HSD17B13 rabbit monoclonal antibody (clone OTI3G2). HSD17B13 protein is a positive control.

Fig 2b HSD17B13 knockdown clones confirmed by ELISA

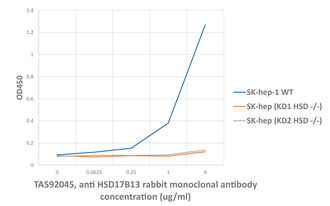
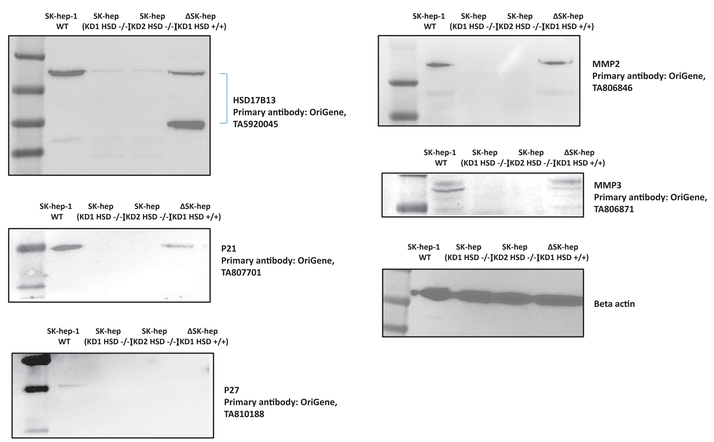


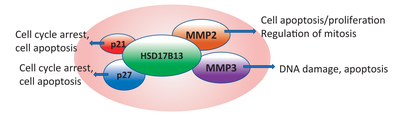
Fig 2b: Analysis HSD17B13 knockdown cells by ELISA. all the lysates were coated on high binding ELISA plates with 30ug/ml. Anti HSD17B13 rabbit monoclonal antibody (OTI3G2) was diluted with variant concentrations.

Fig 3 Analysis of cell cycle related protein expression with HSD17B13 knockdown cells or HSD17B13 overexpressed cells



Results

SK-hep-1 wild type liver cancer cell expresses HSD17B13 and cell cycle related proteins



SK-hep-1 cells with knockdown HSD17B13

HSD17B13 knockdown SK-hep-1 cells transfected with HSD17B13 plasmid

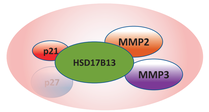


Figure3: Protein expression analysis by Western blot. Total four cell lines were detected. SK-Hep-1 WT is wild type hepatic liver cell line. SK-hep (KD1 HSD-/-) and SK-hep (KD2 HSD-/-) are two HSD17B13 knockdown cell lines by using sgRNA1 primer and sgRNA2 primer respectively. ΔSK-hep (KD1 HSD +/+) is HSD17B13 plasmid DNA transfected SK-hep (KD1 HSD-/-) cells. SK-Hep-1 WT has HSD13B13, p21, p27, MMP2 and MMP3 expression. While HSD17B3 knockdown cells SK-hep (KD1 HSD-/-) and SK-hep (KD2 HSD-/-) don't have HSD13B13, p21, p27, MMP2 and MMP3 expression. With HSD17B13 gene transfection, ΔSK-hep (KD1 HSD +/+) shows overexpressed HSD17B13 and comparable MMP2, MMP3 expression. P21 expression is weaker than wild type cells. No p27 expression was detected.

Fig 4. Flow cytometry for cell cycle analysis

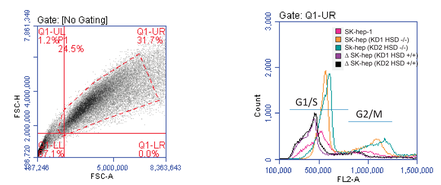


Figure 4: Cell cycle analysis of SK-hep-1 cell lines. Flow cytometry analysis showing the distribution of cells along the different cell cycle phases in wild type cells, knockdown cells and HSD17B3 expression recovered cells. SK-hep-1, HCC cell line, showing G1/S arrest. SK-hep (KD1 HSD-/-) and SK-hep (KD2 HSD-/-) are two HSD17B13 knockdown cell line did not show G1/S accumulation. ΔSK-hep (KD1 HSD +/+) and ΔSK-hep (KD2 HSD +/+) with recovered HSD17B13 expression showing accumulated G1/S phase.

Conclusion

It has been well known that following anti-mitogenic signals or DNA damage, p21 and p27 bind to cyclin-CDK complexes to inhibit their catalytic activity and induce cell-cycle arrest. MMPs family play essential roles in physiological processes such as organogenesis, angiogenesis, apoptosis, cell proliferation and motility. MMP2 and MMP3 have also been found in the nucleus of the cell which may regulate certain nuclear events.

In this study, we are the first to generate the HSD17B13 knockdown cell lines which can be a robust tool for discover more HSD17B13 function in the future. Compare with wild type HCC cell line SK-hep-1, the expression of cell cycle related protein, p21, p27, MMP2 and MMP3 decreases in SK-hep HSD17B13 knockdown cells (HSD-/-). Along with HSD17B13 expression recovery, p21, MMP2 and MMP3 expression are partial/full recovered but p27 expression level does not. This indicates HSD17B13 which has been identified to be a liver enriched, hepatocyte-specific, lipid droplet associate protein has a strong relationship with p21, p27, MMP2 and MMP3 proteins. HSD17B13 play important roles in the progression of HCC lesions. HSD17B13 might be a potential immune-oncology marker for HCC.

The relationship between HSD17B13 and these proteins could be further explored.

Reference

1. Jun Chen, Jian T Zhuo, Fan Yang and etc. "17-beta-hydroxysteroid dehydrogenase 13 inhibits the progression and recurrence of hepatocellular carcinoma." *Hepatobiliary& Pancreatic Diseases Inter., 17* (2018): 220-226.