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Ubiquitin C-terminal Hydrolase 37, a novel predictor for hepatocellular carcinoma recurrence, promotes cell migration and invasion via interacting and deubiquitinating PRP19

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ABSTRACT

Ubiquitin C-terminal hydrolase 37 (UCH37) plays a crucial role in numerous biological processes and is also involved in oncogenesis. In this study, clinicopathologic data showed that UCH37 was over-expressed in hepatocellular carcinoma (HCC) cancerous tissues and was a significant predictor for time to recurrence (TTR). In vitro, we discovered that UCH37 could promote cell migration and invasion. Subsequently, we utilized Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) to identify differentially expressed proteins in UCH37 over-expressing cells compared with the control cells, and found that PRP19, an essential RNA splicing factor, was up-regulated. The relationship between UCH37, PRP19 and the capability of cell migration and invasion was further confirmed. Collectively, this study demonstrated that UCH37 could promote cell migration and invasion in HCC cell lines through interacting and deubiquitinating PRP19, and suggested that UCH37 could be a novel predictor for HCC recurrence after curative resection.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer-related mortality in the world [1]. Its incidence and mortality rates have increased in recent years [2], and the high rate of recurrence or metastasis after curative resection remains one major obstacle for further improving the prognosis of HCC patients [3,4]. Several prognostic biomarkers in HCC have been reported recently [5–7]. Among these, alpha-fetoprotein (AFP) is still the best marker to supervise the recurrence and metastasis in AFP-positive HCC patients after operation [8]. Nevertheless, it is still difficult to predict prognosis in early-stage HCC in the AFP-normal patients [3,9]. Therefore, it is important to determine molecular signatures that define the risk of recurrence and metastatic potential of HCC. And such markers would allow appropriate therapeutic regimens to be applied earlier in the disease course.

Ubiquitin (Ub), a 76-amino-acid polypeptide, is ubiquitously distributed and highly conserved throughout eukaryotic organisms. Over the last few decades, conjugation of Ub and ubiquitin-like proteins to intracellular proteins has emerged as a critical regulatory process in virtually all aspects of cell biology [10–12]. Nevertheless, it is well recognized that protein ubiquitination, like protein phosphorylation, is a highly reversible process that can be regulated in the cell. Deubiquitinating enzymes (DUBs), capable of removing Ub from protein substrates, are also involved in numerous biological processes such as transcriptional regulation, growth and differentiation, and oncogenesis [13–15]. The ubiquitin C-terminal hydrolase (UCH), a subfamily of DUBs, prefers to cleave relatively small protein substrates

Abbreviations: 2D, two-dimensional; ABC, avidin–biotin complex; ACN, acetonitrile; AFP, alpha-fetoprotein; BAP1, BRCA1-associated protein-1; BCLC, Barcelona Clinic Liver Cancer; co-IP, co-immunoprecipitation; CT, computed tomography; DAB, diaminobenzidine; DDR, DNA damage response; DTT, dithiothreitol; DUBs, deubiquitinating enzymes; ESCC, esophageal squamous cell carcinoma; FA, formic acid; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GO, Gene Ontology; HCC, hepatocellular carcinoma; HCD, higher-energy collision dissociation; hINO80, human Ino80 chromatin-remodeling complex; IAM, iodoacetamide; ICLs, interstrand cross-links; iTRAQ, Isobaric Tag for Relative and Absolute Quantitation; KEGG, Kyoto Encyclopedia of Genes and Genomes; MRI, magnetic resonance imaging; MS, mass spectroscopy; NTC, NineTeen associated-Complex; OS, Overall survival; PBS, phosphate-buffered saline; pI, isoelectric point; PI, propidium iodide; SCX, strong cation exchange; SD, standard deviation; shRNA, small hairpin RNA; siRNA, small interfering RNA; TEAB, triethylammonium bicarbonate; TNM, tumor-node-metastasis; TTR, time to recurrence; Ub, ubiquitin; UCH, ubiquitin C-terminal hydrolase; UPP, ubiquitin proteasome pathway; US, ultrasonography; WB, western blotting

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from the C-terminus of Ub [16]. In UCH family, there are four known members: UCH-L1, UCH-L3, UCH37, and BRCA1-associated protein-1 (BAP1).

In our preliminary study, total proteins extracted from one human HCC tissue and one normal liver tissue were separated by two-dimensional (2D) gel electrophoresis, followed by silver staining. Among all protein spots analyzed, about 200 protein spots were found to be significantly altered between the cancerous tissue and the normal tissue. With molecular masses ranging from 25 to 40 kDa and isoelectric point (pI) between 5 and 6, nine protein spots were found in the cancerous tissue, but not in the normal tissue (Supplementary Fig. 1A). We presumed that UCH37 (molecular mass: 37 kDa; pI: 5.23) was one of the differentially expressed proteins. Subsequently, UCH37 mRNA expression was detected by real-time PCR in 22 HCC cases to confirm the hypothesis. Consequently, we found that UCH37 mRNA expression in the cancerous tissue was significantly higher than that in the non-cancerous tissue (Supplementary Fig. 1B). Meanwhile, we chose one of the HCC tissues and one of the normal liver tissues for western blotting and found that UCH37 was highly expressed in the cancerous tissue, but weakly expressed in the adjacent non-cancerous liver and normal liver tissue (Supplementary Fig. 1C). These were inspiring findings and indicated that there was likely relationship between UCH37 and HCC.

UCH37 is a protein of 329 amino acids, which is well conserved from fungi to humans [17]. Previous studies have focused on the deubiquitination mechanism of UCH37. It is responsible for the Ub isopeptidase activity in the 19S proteasome regulatory complex, which is different from other UCH members, showing that neither UCH37 alone nor the UCH37–Adrm1 or UCH37–Adrm1–hRpn2 complexes can hydrolyze Lys48-linked di-ubiquitin efficiently; that rather, their incorporation into the 19S complex is required to enable UCH37 to process large ubiquitin protein conjugates such as di-ubiquitin [17–19]. Further studies have suggested that UCH37 is associated with the human Ino80 chromatin-remodeling complex (hINO80) in the nucleus and can be activated via transient association of 19S regulatory particle- or proteasome-bound hRpn13 with hINO80 [20,21]. Although there is growing evidence that UCH enzymes and human malignancies are closely correlated [22], the biological roles of UCH37 have not been determined.

In the current study, we found out that UCH37 was highly expressed in HCC cancerous tissues and explored the predictive value of UCH37 for HCC recurrence after curative resection. Furthermore, we discovered that UCH37 could promote cell migration and invasion in HCC cell lines through interacting and deubiquitinating PRP19, an essential RNA splicing factor.

2. Material and methods

2.1. Patients and follow-up

Tumor specimens used in the current study were obtained from 90 HCC patients who underwent curative resection at Liver Cancer Institute, Zhongshan Hospital, Fudan University from October 1, 2006 to December 31, 2008. The inclusion and exclusion criteria of the patients included (1) having a distinctive pathologic diagnosis of HCC; (2) having no anti-cancer treatment before liver resection; (3) having curative liver resection; (4) having suitable formalin-fixed, paraffin-embedded tissues; and (5) having a complete clinicopathologic and follow-up data.

Curative resection was defined as: (1) complete resection of all tumor nodules and the surgical free margin of more than 5 mm by pathological examination; (2) having no cancerous thrombus found in the portal vein (main trunk or two major branches), hepatic veins, or bile duct; and (3) having no extrahepatic metastasis found. Tumor differentiation was defined according to the Edmondson grading system. Tumor staging was defined according to the 6th edition of tumor-node-metastasis (TNM) classification of Unio Internationale Contra Cancrum. HCC staging was defined according to the Barcelona Clinic Liver Cancer (BCLC) staging system. The clinicopathologic characteristics of 90 patients were summarized in Supplementary Table 1.

All patients were followed up every 2 months during the first post-operative year and at least every 3–4 months afterward. Follow-up was finished on March 31, 2011. Most patients died of intrahepatic recurrence, distal metastasis, or complicated liver cirrhosis. All patients were monitored prospectively by serum AFP, abdomen ultrasonography (US), and chest X-ray every 1–6 months, according to the postoperative time. For patients with test results suggestive of recurrence, computed tomography (CT) and/or magnetic resonance imaging (MRI) were used to verify whether intrahepatic recurrence and/or distal metastasis had occurred. A diagnosis of recurrence was based on typical imaging appearance in CT and/or MRI scan and an elevated AFP level. Overall survival (OS) was defined as the interval between surgery and either death or the last observation taken. Time to recurrence (TTR) was measured from the date of resection until either detection of recurrent tumor or the last follow-up assessment. The current study was approved by the Institutional Ethics Committee of Zhongshan Hospital, Fudan University. All patients provided written informed consent.

2.2. Cells and reagents

Purified pcDNA3.1-UCH37 plasmid was obtained as a kind gift from Professor Xingzhong Wu (Department of Biochemistry, Shanghai Medical School, Fudan University). L02 and Huh7 cell lines were purchased from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China); supervision anti-rabbit or anti-mouse detection reagents (HRP), from Kangwei Biotechnology (Beijing, China); mouse anti-UCH37 antibody, mouse anti-PRP19 antibody and mouse anti-Ub antibody, from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit anti-PRP19 antibody, from Abcam Biotechnology (USA); mouse anti-Flag and anti-GAPDH antibodies, from Kangwei Biotechnology (Beijing, China); ECL Western Blotting Substrate System, from Pierce (Rockford, IL, USA); Lipofectamine 2000 and FITC-AnnexinV/propidium iodide (PI) cell apoptosis kit, from Invitrogen (USA); Transwell with 8-μm pore polycarbonate membrane, from Millipore (USA); crystal violet, from Sigma (St. Louis, USA); Matrigel, from BD Biosciences (USA); MG132, from Merck (Germany); and cycloheximide, from Beyotime Biotechnology (Shanghai, China).

2.3. Cell culture and stable transfectants

Respectively, L02 and Huh7 cells were cultured in RPMI medium 1640 (Thermo, USA) and DMEM (Thermo, USA), supplemented with 15% fetal bovine serum (FBS) (GIBCO, Austria) at 37 °C in a humidified atmosphere containing 5% CO_2. The cell line stably expressing UCH37 was generated by the retroviral infection of L02 cells. The target sequence for the small hairpin RNA (shRNA) for UCH37 is 5′-CCTGTTAATGGGAGACTGTAT-3′. A retrovirus-expressing shRNA specific to UCH37 was infected into Huh7 cells. Briefly, a retroviral vector containing either human UCH37 cDNA with an N-terminal Flag-tag or a specific shRNA, pHCMV-G and pCMV-dR8.9 were co-transfected into 293 T cells, and the viral supernatants were collected to infect L02 or Huh7 cells. Monoclonal cells were then selected, cloned, and screened for UCH37 over-expression or down-regulation.

2.4. Real-time quantitative PCR analysis

The mRNA quantity of specific genes, calculated using the ΔΔCt method, was normalized against β-actin. Real-time PCR amplification involved the use of an ABI Prism 7500 sequence detector (Applied Biosystems) and SYBR reagent (Takara, Japan). All the measurements