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UBE2S enhances the ubiquitination of p53 and exerts oncogenic activities in hepatocellular carcinoma

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ABSTRACT

Ubiquitin-conjugating enzyme E2S (UBE2S) plays pivotal roles in the progression of human cancers. However, its clinical significance and role in hepatocellular carcinoma (HCC) remain unknown. Here, we show that UBE2S is upregulated in HCC and exhibits oncogenic activities via enhancing the ubiquitination of p53. Increased expression of UBE2S was significantly correlated with higher serum AFP level, higher pathological grade, advanced TNM stage, larger tumor size, vascular invasion and unfavorable patient survivals in two independent cohorts containing a total of 845 patients with HCC. Multivariate analyses by cox regression model suggested UBE2S as an independent factor for overall survival. *In vitro* experiments demonstrated that UBE2S overexpression promoted, whereas UBE2S knockdown suppressed cell proliferation and migration via modulation of p53 signaling pathway. Ectopic expression of UBE2S upregulated the expression of p53 and its downstream effectors, such as p21 and Cyclin D1. Mechanistically, UBE2S enhanced the ubiquitination of p53 protein to facilitate its degradation in HCC cells. Re-expression of p53 partially attenuated the UBE2S-promoted malignant phenotypes. Collectively, our study provides compelling evidence that UBE2S is a potential prognostic factor and functions as an oncogene in HCC.

1. Introduction

Hepatocellular carcinoma (HCC) ranks the fifth most prevalent cancer, and the third as a cause of cancer death worldwide [1]. The morbidity of HCC has been increasing in economically developed regions, including Japan, Western Europe and the United States in recent decades [2], making HCC as a global threat to human lives. In view of that the poor prognosis of HCC, with a median survival time of 4 months, and that the accuracy and reproducibility of markers currently used in clinic to predict survival after surgical resection remain either unsatisfactory or unclear, efforts have been made to uncover the mechanism of HCC progression. Identification of biomarkers with potent prognostic or therapeutic value is of immense importance to develop novel strategies for the clinical management.

Ubiquitin-conjugating enzyme E2S (UBE2S, also known as E2-EPF), an E2 enzyme [3], coupled with ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C) to elongate K11-linked polyubiquitin chain on substrates for 26 S proteasome-mediated degradation to play pivotal roles in cell division [4–8]. The association of UBE2S and APC/C was inhibited by Emi1 [4], Emi2 [9] and phosphorylation of CDC20 [10]. UBE2S is responsible for the ubiquitin-mediated proteasomal degradation of Sox2 in mouse embryonic stem cells to control the cell differentiation [11]. The critical role of UBE2S in regulating cell cycle, cell differentiation and DNA repair inevitably implicates its involvement of tumorigenesis [12,13]. Aberrant expression of UBE2S has been reported in oral squamous cell carcinoma [14], cervical cancer [15] and renal cancer [16]. High UBE2S expression, co-expressed with the well-known breast cancer markers MKI67 and AURKA, was associated with poor survival in breast cancer [17]. UBE2S mediated the malignant

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phenotypes in colorectal cancer via stabilizing β-Catenin through directly interaction with β-Catenin to ubiquitinate its K19 residue via K11 linkage [18]. Knockdown of UBE2S impaired the tumor growth and antagonized the chemo-resistance in glioblastoma [19]. However, its biological function in HCC remain unclear.

In this study, the expression of UBE2S and clinical significance were determined in two independent cohorts containing a total of 845 patients with HCC. The role of UBE2S in HCC cell proliferation and migration, as well as the underlying mechanism, was investigated. Our data indicate that UBE2S is upregulated in HCC and exhibits oncogenic activities via enhancing the ubiquitination of p53.

2. Materials and methods

2.1. Patients and specimen

A cohort containing 486 paraffin-embedded HCC samples was obtained from May 2005 to December 2012 in Shenzhen People's Hospital (SPH cohort). The mean age of patients was 49.5 years old (ranging from 18 to 79). The median follow-up was 26.5 months. Another cohort consisting of 49 cases with portal vein embolus and 35 fresh specimens was collected. Informed consent was obtained. None of the patients received chemotherapy or radiotherapy before surgery. All samples were anonymous. The use of human samples was approved by the Research Ethics Committee of Second Clinical Medical College of Jinan University and carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.2. Cell lines and transfection

HCC cell lines (Bel-7402, Bel-7404 and MHCC-97H) purchased from the Cell Resource Center, Chinese Academy of Science Committee (Shanghai, China) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT) in a humidified incubator at 37 °C and 5% CO2. The cells were stably transfected with UBE2S overexpression vector or shRNAs with Lipofectamine 2000. Stable cell lines were established by G418 screening for 2 weeks. The shRNA for UBE2S were obtained from Santa Cruz Biotechnology (sc-97109-SH).

2.3. Quantitative real-time PCR

Complementary DNA was synthesized from the total RNA, using the PrimeScript RT reagent Kit (TAKARA, USA). qRT-PCR was performed with SYBR Premix ExTaq (TAKARA, USA). The expression of the endogenous 18s was used as control for the normalization of the relative expression of UBE2S. The $-\Delta Ct$ was calculated. Conditions for RT-PCR was set as the following: 95 °C for 10 min, 40 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and a final extension of 10 min at 72 °C. The primers were as following: UBE2S, forward: 5′- GACCGAAGAACGCAGGAAG-3′ and reverse: 5′-GTGCGGGGGTAGGTTCTC-3'; β-actin, forward: 5′-TGGCACCCAG-CACAATGAA-3′ and reverse: 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3'.

2.4. MTT

Stable cells were cultured in 96-well plates for 5 days. 20 μl of MTT (5 mg/ml) was added into the wells for 3 h. The formazan crystals were dissolved in DMSO (150 μl/well). The absorbance at 490 nm of each sample was measured. The cell growth rate was calculated.

2.5. Colony formation

Stable cells were constructed. Cells were collected and seeded in 6-well plates at a density of 1.0×103 per well and then incubated at 37 °C for 10 days. Colonies were fixed with methanol and stained with 0.1% crystal violet and counted.

2.6. Western blot

Proteins extracted from HCC fresh tissue or cells with various treatments were fractionated by SDS-PAGE, transferred to PVDF membrane, and then incubated with a primary specific antibody for UBE2S (1:1000, #11878, Cell signaling technology), p53 (1:1000, sc-126, Santa-cruz), p21(1:1000, #2947, Cell signaling technology), Cyclin D1 (1:1000, #2978, Cell signaling technology), 14-3-3σ(1:1000, #7413, Cell signaling technology) and β-actin (1:1000, #4970, Cell signaling technology) in 5% of non-fat milk, followed by a horse radish peroxidase (HRP)-conjugated anti-rabbit/mouse second antibody. ECL detection reagent (Amersham Life Science, Piscataway, NJ, USA) was used to visualize the results.

2.7. Immunohistochemistry and scoring

TMA sections with a thickness of 4 μm were dewaxed in xylene and graded alcohols, hydrated, and washed in phosphate buffered saline (PBS). After pretreatment in a microwave oven, endogenous peroxidase was inhibited by 3% hydrogen peroxide in methanol for 20 min, followed by avidin-biotin blocking using a biotin-blocking kit (DAKO, Germany). Slides were then incubated with UBE2S (1:500, #11878, Cell signaling technology) or p53 (1:500, sc-126, Santa-cruz), overnight in a moist chamber at 4 °C, washed in PBS, and incubated with biotinylated goat anti-rabbit antibody. Slides were developed with the Dako Liquid 3, '3-diaminobenzidine tetrahydrochloride (DAB) + Substrate Chromogen System and counterstained with hematoxylin.

IHC evaluation was determined by semi-quantitative IHC detection, using the H-score method. The percentage of positively-stained cells was scored as "0" (0%), "1" (1%–25%), "2" (26%–50%), "3" (51%–75%), "4" (76%–100%). Intensity was scored as "0" (negative staining), "1" (weak staining), "2" (moderate staining), and "3" (strong staining). The percentage score was multiplied by the staining intensity score. For each case, 1000 cells were randomly selected and scored. The scores were independently decided by 2 clinical doctors. The median of UBE2S IHC score, which was 4.5, was chosen as the cutoff value to identify high and low expression groups.

2.8. Statistical analysis

Continuous variables were expressed as a mean with SEM and analyzed using the *Student t*-test (2-tailed). Kaplan-Meier analysis (the log-rank test) was used for survival analysis and univariate analysis. The Cox proportional hazards regression model was used to evaluate the independent prognostic value of UBE2S in HCC. The *P* value less than 0.05 was considered to be statistically significant.

3. Results

3.1. UBE2S expression is increased in HCC

To determine the expression of UBE2S in HCC, 35 pairs of HCC fresh tissues were collected. qRT-PCR results showed that UBE2S mRNA was significantly upregulated in HCC tissues, compared with the corresponding nontumorous tissues (Fig. 1A). Consistently, the

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