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Alternative splicing variants of human Fbx4 disturb cyclin D1 proteolysis in human cancer

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ABSTRACT

Fbx4 is a specific substrate recognition component of SCF ubiquitin ligases that catalyzes the ubiquitination and subsequent degradation of cyclin D1 and Trx1. Two isoforms of human Fbx4 protein, the full length Fbx4α and the C-terminal truncated Fbx4β have been identified, but their functions remain elusive. In this study, we demonstrated that the mRNA level of Fbx4 was significantly lower in hepatocellular carcinoma tissues than that in the corresponding non-tumor tissues. More importantly, we identified three novel splicing variants of Fbx4: Fbx4γ (missing 168–245nt of exon1), Fbx4δ (missing exon6) and a N-terminal reading frame shift variant (missing exon2). Using cloning sequencing and RT-PCR, we demonstrated these novel splice variants are much more abundant in human cancer tissues and cell lines than that in normal tissues. When expressed in Sk-Hep1 and NIH3T3 cell lines, Fbx4β, Fbx4γ and Fbx4δ could promote cell proliferation and migration *in vitro*. Concordantly, these isoforms could disrupt cyclin D1 degradation and therefore increase cyclin D1 expression. Moreover, unlike the full-length isoform Fbx4α that mainly exists in cytoplasm, Fbx4β, Fbx4γ, and Fbx4δ locate in both cytoplasm and nucleus. Since cyclin D1 degradation takes place in cytoplasm, the nuclear distribution of these Fbx4 isoforms may not be involved in the down-regulation of cytoplasmic cyclin D1. These results define the impact of alternative splicing on Fbx4 function, and suggest that the attenuated cyclin D1 degradation by these novel Fbx4 isoforms provides a new insight for aberrant cyclin D1 expression in human cancers.

1. Introduction

Ubiquitin (Ub)-mediated proteolysis is an important means of regulating gene expression and has a pivotal role in the control of various cellular processes [1]. Ubiquitination is catalyzed via a triple-enzyme cascade including an E1 (Ub-activating enzyme), E2 (Ub-conjugating enzyme), and E3 (Ub ligase). Once ubiquitinated, proteins are rapidly hydrolyzed by the 26S proteasome. The substrate specificity is largely conferred by E3 ligases, and the interaction between substrates and E3 is crucial for the regulation of Ub-mediated protein turnover [2].

Skp-cullin-F-box (SCF) Ub ligases are multi-subunit complexes that mediate specific ubiquitination of distinct substrates [3]. Substrate specificity is decided by the F-box protein subunit of the complex. Recently, the Ub ligase SCF^{Fbx4} has been identified as a new E3 responsible for the ubiquitination and subsequent degradation of the cell cycle regulator cyclin D1 and telomeric DNA-binding protein Pin2 (also known as Trf1) [4,5]. Fbx4 belongs to the FBXO subfamily of F-box proteins in which the substrate-binding motif has not been identified yet [6]. Structurally, Fbx4 has several identified domains essential for its function in SCF complex: N-terminal dimerization domain (D domain), F-box domain, linker domain and C-terminal substrate-binding domain (G domain) [6–9]. Structural and biochemical analyses have revealed that the interaction between linker domain and G domain is crucial for the head-to-tail dimerization configuration of Fbx4, which is required for substrate binding and ubiquitin transfer [9].

Fbx4 recognizes its two known substrates cyclin D1 and Pin2 in two very different manners. Fbx4-mediated ubiquitination of cyclin D1 depends on its phosphorylation at Thr-286 residue, as well as an interaction with αB-crystallin, a small heat-shock protein, whereas that of Pin2 does not require phosphorylation [4,5,10]. Inhibition of Fbx4 activity results in accumulation of nuclear cyclin

Abbreviations: Fbx4, F-box only protein 4; Ub, ubiquitin; SCF, Skp-cullin-F-box; HCC, hepatocellular carcinoma.

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D1 and oncogenic transformation which appears at least in part to account for overexpression of cyclin D1 in human cancers [5,11], and mutations in the Fbx4 subunit of SCF^{Fbx4} were found to be associated with human primary esophageal carcinoma [12,13]. Therefore, the regulation of SCF^{Fbx4} activity is considered to be very important to sustain cyclin D1 level in normal cells, and impairment of SCF^{Fbx4} function is a critical mechanism underlying cyclin D1 overexpression in human cancers.

To date, two Fbx4 isoforms (named Fbx4α and Fbx4β in this paper) created by alternative splicing have been identified, but the roles these two isoforms play in SCF^{Fbx4} activity remain incompletely understood. Although the alternative splicing has been assigned as a regulatory mechanism for F-box protein [14], the specific differences of Fbx4 splicing profiles in cancer tissues remain elusive. In this study, we identified the presence of additional oncogenic splicing variants of Fbx4 in human tissues and these novel Fbx4 splicing variants are much more abundant in tumor tissues and cell lines than that in normal tissues. The attenuated cyclin D1 degradation by these novel Fbx4 isoforms *in vitro* provides a new insight for aberrant cyclin D1 expression and tumor development in human cancer.

2. Materials and methods

2.1. Cell lines and tissues

Human liver cancer cell lines SNU182, SNU449, HepG2, SK-Hep1 and mouse embryo fibroblast cell line NIH3T3 were purchased from ATCC (American Type Culture Collection; Manassas, VA, USA). The esophagus carcinoma cell lines KYSE450, KYSE510, SEG-1, BIC-1 and gastric cancer cell line NUGC3 were gifts from Professor Mingzhou Guo (Chinese PLA General Hospital).

A total of 40 pairs of HCC tissues and non-tumor tissues were provided by the Affiliated Oncology Hospital of Zhengzhou University. Both tumor samples and non-tumor samples were confirmed histologically. The usage of human samples in this study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by the Ethics Committee of Peking University Health Science Center.

2.2. Reverse transcriptase polymerase chain reaction (RT-PCR)

Quantitative real-time RT-PCR (qRT-PCR) was carried out using SYBR Green on a LightCycler 480IIreal-time PCR detection system (Roche, Germany) according to the manufacturer's instruction. To quantify novel Fbx4 isoforms, semi-quantitative PCR was performed with primers specific to each isoform, and the products were analyzed on 2% agarose gel. The primers used for RT-PCR are summarized in Table S1.

2.3. Clone-sequencing of Fbx4 mRNA composition

To clone Fbx4 mRNA, PCR reactions were performed with primers CDS1-F/R and CDS2-F/R (Table S1) by using cDNA from normal tissues and tumor cell lines, respectively. PCR products were purified and cloned into TA vector and transformed into *Escherichia coli* DH5α competent cells. Cell clone was randomly picked up and sequenced.

2.4. Plasmid construction

To construct vectors expressing Fbx4 isoforms, cDNA fragments from normal liver tissues were prepared by RT-PCR with specific primers (Table S1). The cDNA of Fbx4 isoforms was then cloned into pcDNA™3.1/Myc-His (−) vector (Invitrogen, Carlsbad, California). All expressing vectors were verified by DNA sequencing and Western blot assay.

2.5. Colony formation assay

Colony formation assay were performed using a soft agar kit (GenMed Scientifics, Inc., Arlington, USA) in 6-well plates according to the manufacturer's instructions. Colonies were photographed and counted under a microscope with a digital camera.

2.6. Transwell assay

The transwell system was used to explore cell's migration. A total of 7500 cells suspended with 150 μL of FBS-free medium were added into the inside compartment of the transwell insert. After 48-h incubation, the migrated cells were stained with 0.1% crystal violet and quantified under a light microscope, with at least three individual fields per insert.

2.7. Immunofluorescence assays

Cells were permeabilized with Methanol:Acetone (1:1), washed with PBS and incubated in primary anti-Myc antibody for 2 h. After washing and application of the secondary FITC-conjugated secondary antibody, slides were stained using Hoechst 33258 dye and analyzed by fluorescence microscopy (LEICA).

2.8. Subcellular fractionation analysis

NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Pittsburgh, PA) were used to get cytoplasmic extract and nuclear extract, respectively, according to the manufacturer's recommendation.

2.9. Immunoprecipitation (IP) and Western blot assays

For IP assay, cells were lysed with IP buffer. The cell lysates were incubated with the primary antibody and then immunocomplexes were bound to protein-G Sepharose 4B (Pharmacia, Sweden). After extensive washing, the precipitates were analyzed by Western blot. Western blot analysis was performed as described [13]. The antibodies used for IP and Western blot analysis were listed in Table S2.

2.10. Statistical analysis

For statistical analyses, the difference between two groups was analyzed by 2-tailed Student *t* test using GraphPad Prism software. In all cases, a *P* value of less than 0.05 was considered significant.

3. Results

3.1. Fbx4 is down-regulated in HCC tissues

To examine whether Fbx4 is down-regulated in HCC tissues, we performed qRT-PCR to measure Fbx4 mRNA level in 40 pairs of HCC tumor and adjacent non-tumor tissues. The result showed that Fbx4 mRNA level in tumor tissues was significantly lower than that in adjacent non-tumor tissues ($P = 0.0238$) (Fig. 1A). To further confirm the reduced expression of Fbx4 in HCC, 9 pairs of HCC tissues were used for Western blot assay to analyze Fbx4 protein level. Consistent with the lower Fbx4 mRNA level, lower Fbx4 protein level was detected in 7 of 9 HCC tumor tissues, as compared to their matched non-tumor tissues (Fig. 1B).