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hZG16, a novel human secreted protein expressed in liver, was down-regulated in hepatocellular carcinoma

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Abstract

Here we reported a novel human secreted protein named as hZG16, with a Jacalin domain. Evolution analysis through comparing with the orthologs of other organisms suggested that *ZG16* is a conserved gene under the purifying selection ($d_N/d_s < 1$) in the evolution. Interestingly, Northern and dot blot analyses showed that *hZG16* were highly expressed in adult liver, not in fetal liver, and moderately in gut, including jejunum, ileum, and colon, in which the tissue expression pattern of *hZG16* was significantly dissimilar to that of mouse and rat orthologs that were uniquely expressed in spleen and pancreas, respectively. Unexpectedly, *hZG16* was markedly down-regulated in hepatocellular carcinoma (HCC) as indicated by RT-PCR, Northern blot analysis and immunohistochemistry staining. However, the tunicamicin treatment and pulse-chase experiments showed that hZG16 protein had a similar molecular function with rZG16 that take part in glycoproteins' secretion in a bus mode.

Keywords: hZG16; Jacalin domain; Secreted protein; Liver-specific expression; Purifying selection

Rat ZG16 (rZG16), a zymogen-processing protein, was first found to be highly expressed in pancreas, colon, and duodenum [1], where the protein were localized in the zymogen granule of pancreas. The previous reports indicated that rZG16 took part in the formation of zymogen granule by mediating the digested enzymes to the zymogen granule membrane in pancreatic acinar cells [2,3]. Moreover, the mouse homologue of the zymogen-processing protein ZG-16 was also highly expressed in the pancreas but sharply down-regulated early in the course of injury [4], implying that mZG16 could be involved in other physiological activity.

However, all ZG16 proteins have a Jacalin domain. A variety of proteins containing this domain are lectins, such as Jacalin protein from the seed of the *Artocarpus heterophyllus* (jackfruit), which is specific for galactose and can bind to the T-antigen [5]. The domain is also found in the salt-stress induced protein from rice and animal prostatic spermine-binding protein that will be mentioned next.

Recently, hZG16, the human homologue of rZG16 was screened out as a novel secreted protein in our lab. Interestingly, hZG16 exhibited the different tissue expression pattern, which was significantly dissimilar to that of mouse and rat orthologs. Moreover, *hZG16* was markedly down-regulated in hepatocellular carcinoma (HCC) as indicated by RT-PCR, Northern blot analysis and immunohistochemistry staining. The tissue expression patterns of ZG16 in different organisms implied that the genes could be involved in the distinct functions in different organs. Actually, the pulse-chase experiments showed that hZG16 protein took part in several glycoproteins' secretion.

Materials and methods

Bioinformatics analysis. The novel secreted proteins hZG16 was screened out as the previous described by Zhou et al. [6] The online softwares SignalP v.2.0 (http://www.cbs.dtu.dk/services/SignalP-2.0/),

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PSORT II (http://psort.nibb.ac.jp/), and SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) were here used to predict the novel secreted proteins derived from the public protein database. The human ZG16 was further analyzed with BLAST (http://www.ncbi.nlm.nih.gov/blast/) and SMART (http://smart.embl-heidelberg.de/) and ENSEMBL (http://www.ensembl.org). Alignment and phylogenies were inferred by computer program CLUSTALW, Gendoc, and MEGA2. SNAP (http://www.hiv.lanl.gov/content/hiv-db/SNAP/WEBSNAP/SNAP.html) was used to calculate the evolutionary ratio of d_S/d_N.

RNA extraction, RT-PCR and cDNA cloning. Total RNA was extracted with TRIZOL reagent (Life Technologies Inc., Gaithersburg, MD) according to the manufacturer's instructions. RNA was resuspended in diethylpyrocarbonate-treated water and quantitated by spectrophotometer. Reverse transcription was carried out at 42 °C for 1 h in a 25-μL reaction mixture that contained 3 μg of total RNA, 10 pmol of oligo-dT, and 200 U of M-MLV reverse transcriptase (Promega, Madisn, USA). The entire open reading frame (ORF) of *hZG16* was amplified by reverse transcription-polymerase chain reaction (RT-PCR) from human liver with PCR primers: hZG16-3.1A-F-P (5′-CCGGATCCATGTTGACAGTCGCTCTCCT-3′) and hZG16-3.1A-R-P (5′-CCCGGTACCGCATCTGCTGCAGCTAG TG-3′). PCR was carried out in PE9700 thermal cycler under the conditions of 94 °C for 4 min, 35 cycles of 94 °C for 40 s, 60 °C for 30 s, 72 °C for 50 s, and a final extension at 72 °C for 7 min. For mammalian cell expression, the PCR product was inserted into pcDNA3.1-Myc-HisA(−) vector (Invitrogen) to generate a recombinant plasmid pcDNA3.1A-hZG16, encoding fusion protein hZG16 with a Myc-His-tag at the C-terminus. The β-actin was used as an internal control, and the PCR primers are β-actin-F-P (5′-TCACCCACACTGTGCCCATCTACGA-3′) and β-actin-R-P (5′-CAG CGGAACCGCTCATTGCCAATGG-3′). PCR product was detected by electrophoresis on a 2% agarose gel.

Northern blot analysis. Total RNA (15 μg) was loaded per lane on a 1.2% denatured formaldehyde agarose gel. After electrophoresis, RNA was transferred to Hybond-N nylon membrane (Amersham Pharmacia Biotech, Ltd., Buckinghamshire, UK) and immobilized by ultraviolet cross-link with probes ([^{32}P]-labelled) prepared with Random Primer DNA Labeling Kit (TaKaRa Inc., DaLian, China) using partial hZG16 (amino acids: 19–167) fragment or using the mZG16 full ORF fragment. Homemade nylon membrane, Human Mulitple Tissue Northern Blot (Clontech) and Dot blot membrane consisting of 46 tissues (BioChain Institute) were hybridized according to the manufacturer's instructions. After hybridization, the membranes were washed in 2× sodium saline citrate, 0.05% sodium dodecyl sulfate (SDS) for 30 min at room temperature and then in 0.1× sodium saline citrate, 0.1% SDS for 30 min at 50 °C and visualized by autoradiography.

Cell transfection and Western blot analysis. CHO cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) in 5% CO_2 at 37 °C. Plates seeded in 1.5×10^5 cells/ml were grown overnight. The expression plasmid pcDNA3.1-hZG16-Myc was transfected transiently into CHO cells with LipofectAMINE reagents (Gibco-BRL), according to the manufacturer's instructions. After 72 h, the cultured supernatant of transfected CHO cells was collected, and the cells were washed two times with cold PBS (pH 7.4) and lysed with single detergent lysis buffer (50 mmol/l Tris–Cl (pH 8.0), 150 mmol/l NaCl, 100 μg/ml PMSF, and 1% Triton X-100) on ice for 30 min. The cultured supernatants and the cell lysates were purified using TALON metal affinity resins (Clontech) according to the manufacturer's instructions. Samples were separated by 15% SDS–polyacrylamide gel electrophoresis (PAGE), followed by transferring onto PVDF membrane (Amersham Life Science) electrophoretically. After blocking in phosphate-buffered saline containing 2% bovine serum albumin, 3% degreased powdered milk, and 0.1% Tween 20 overnight at 4 °C, the membrane was incubated with monoclonal antibody against human c-myc (9E10) (Clontech) or anti-hZG16 polyclonal antibody at room temperature for 2 h, followed by incubation with a horseradish peroxidase-linked secondary antibody (Gibco-BRL) at room temperature for 2 h. The signals were detected using the enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech, Ltd.).

Purification of recombinant protein and production of polyclonal antibody. *Escherichia coli* strain BL21 was transformed with pGEX-hZG16 (19–167 aa) and grown at 37 °C in 2× YT medium containing 100 μg/mL ampicillin to reach $OD_{600} = 0.6$. After additional 6 h at 30 °C with isopropylthio-α-D-galactoside at a concentration of 200 μmol/L, cells were harvested and suspended in 1× phosphate-buffered saline containing 5 mmol/L dithiotreitol and 100 μg/mL phenylmethylsulfonyl fluoride. After sonication, the glutathione-*S*-transferase (GST)-hZG16 fusion protein was purified using GST affinity columns (Amersham Pharmacia Biotech, AB, Uppsala, Sweden) according to the manufacturer's protocol. Three rabbits were immunized three times with standard procedures using 2 mg of (GST)-hZG16 (19–167aa) as antigen for injection. The antiserum then was purified using Protein G–Sepharose 4 Fast Flow (Amersham Pharmacia Biotech, AB) and GST-beads according to the manufacturer's protocol.

Immunofluorescence microscopy. Cells cultured on polylysine-treated slides were transfected with 1 μg of plasmid pcDNA3.1-hZG16 and pEYFP-Golgi (Clontech) using LipofectAMINE (Gibco-BRL). After 60 h, transfected cells were fixed with 2% paraformaldehyde (PFA)/Triton X-100 in PBS on ice for 30 min, after blocking in phosphate-buffered saline containing 5% BSA overnight at 4 °C, cells were immunostained with monoclonal antibodies to human c-myc (9E10) (Clontech) at room temperature for 2 h, followed by incubation with cy5-conjugated anti-mouse immunoglobulin G antibody (Biological Detection Systems, Inc., Pittsburgh, PA, USA) at room temperature for 2 h. The slides were viewed with LSM 510 META Laser Scanning Confocal Microscope (Zeiss).

Immunocytochemistry staining. Human liver of HCC patients was excised and frozen, then sectioned with a cryostat at 5 μm. The slides were fixed with iced acetone for 30 min followed by incubation with 3% H_2O_2 in methanol at 37 °C for 30 min to quench endogenous peroxidase activity. After blocking in 20% goat serum in phosphate-buffered saline at 37 °C for 30 min, the slides were incubated with anti-hZG16 polyclonal antibody at 37 °C for 2 h, then 4 °C overnight, followed by incubation with a horseradish peroxidase-conjugated anti-rabbit antibody (Dako Japan Ltd., Kyoto, Japan) at 37 °C for 1 h. The signals were detected using Diaminobenzidine Substrate Kit (Vector Laboratories, Inc., Burlingame, CA).

Pulse-chase experiments. Tansey Lab Protocols (http://tanseylab.cshl.edu/pdf/PC_mammals.pdf) were referenced for our pulse-chase experiments. In briefly, HeLa cells were seeded in 35 mm dish with ~50% confluent and grown overnight in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, MD) supplemented with 10% (v/v) fetal bovine serum (FBS) by 5% CO_2 at 37 °C. And plasmid pcDNA3.1A-hZG16 was transfected with LipofectAMINE reagents (Gibco-BRL), according to the manufacturer's instructions. Plasmid pcDNA3.1A was used as negative control. After 48 h transfected, the cells were washed twice with warm PBS, starved with 600 μl warm DMEM (met/cys-free)/5% FCS for 30 min at 37 °C, and then were added 12 μl [^{35}S]/dish(10 μCi/μl)for the pulse time: 40 min at 37 °C. Radioactive medium was removed, cells were washed twice with warm PBS and added with 600 μl/dish warm DMEM 5% FCS for the different chase time. At the end of each chase, the medium was collected and glycoproteins were purified with Con A–Sepharose (Amersham Biosciences Limited) as Neve et al. [7] reported.

Tunicamycin and glucose treatment. Tunicamycin treatment was according to the method of Zhou et al. [6]. The stable transfected cell line with exogenous hZG16 was seeded at 5×10^5/35-mm dish and cultured for 24 h, and then the medium was replaced with fresh medium containing tunicamycin (Sigma, St. Louis, MO) (2.0 μg/ml) or fresh medium without glucose and cultured for additional 24 h. The proteins purified from CL and CM were analyzed with Western blotting.

Results

ZG16 is an evolutionally conserved gene under purifying selection

The prediction of novel human secretory proteins was carried out in our group utilizing public human protein