

A Novel Liver-Specific Zona Pellucida Domain Containing Protein That Is Expressed Rarely in Hepatocellular Carcinoma

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We currently identified a liver-specific gene that encodes a novel zona pellucida (ZP) domain-containing protein named liver-specific ZP domain-containing protein (LZP). The full-length complementary DNA (cDNA) of human LZP has 2,255 bp with a complete open reading frame (ORF) of 1,635 bp. The gene is localized on chromosome 10q21.3 and spans 40 kb with 9 encoding exons and 8 introns. The deduced protein sequence has 545 amino acid residues, with an N-terminal signal peptide followed by 3 epidermal growth factor (EGF)-like domains and a ZP domain in C-terminal section. Interestingly, human LZP is expressed specifically in liver out of 23 tissues examined, and its mouse counterpart was detected at very early stage during embryo development. Moreover, LZP can be secreted into blood, albeit the protein was localized mainly on the nuclear envelop of hepatocytes. Most importantly, LZP is down-regulated in hepatocellular carcinoma (HCC) and HCC cell lines; meanwhile, the decreased level of *hLZP* messenger RNA (mRNA) could, at least in some HCC samples, be related to the methylation status of the putative LZP promoter. However, overexpression of *hLZP* in HCC cell line SMMC-7721 and human liver cell line L02 by stable cell transfection did not inhibit cell growth, implying that the down-regulation of *hLZP* in HCC might be a consequence of the dedifferentiation involved in hepatocarcinogenesis. In conclusion, these data suggest that LZP is a liver-specific protein involved possibly in hepatocellular function and development, and the protein could be used as potential negative biomarker for HCC pathologic diagnosis. (HEPATOLOGY 2003;38:735-744.)

Hepatocellular carcinoma (HCC) is the major malignancy associated with liver and is one of the most common malignant tumors in the world, especially in Asia, Africa, and southern Europe. Of

the ~350,000 new HCC cases per year, two thirds occur in Asia, in which China accounts for 50%.¹ Risk factors for hepatocarcinogenesis include infection with hepatitis B virus (HBV), hepatitis C virus, alcohol-induced cirrhosis, exposure to chemical carcinogens, and other factors associated with chronic inflammatory and hepatic regenerative changes.^{1,2} Oncogenes such as *c-myc* and *cyclin D1* and tumor suppressor genes such as *p53*, *Rb*, and *p16^{INK4a}* have been found to be involved in pathogenesis of HCC.³⁻⁷ Mutations in *β -catenin* and *AXIN1* gene have been detected and appear to play an important role in hepatocellular carcinogenesis.^{8,9} A putative tumor suppressor gene *LPTS* localized at chromosome 8p23, which has a high-frequency loss of heterozygosity region in HCC, was considered a candidate involved in hepatocarcinogenesis.¹⁰ However, the molecular mechanisms involved in malignant transformation of hepatocytes are still largely unknown.

Recently, many transcriptome approaches, such as expressed sequence tag (EST) sequencing, complementary DNA (cDNA) microarray, and oligonucleotide chip, were used for studying the molecular mechanism of HCC.¹¹⁻¹³ In our previous work, comprehensive characteristics of HBV-positive HCC were described by com-

Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; EST, expressed sequence tag; cDNA, complementary DNA; RT-PCR, reverse transcription-polymerase chain reaction; ZP, zona pellucida; LZP, liver-specific ZP domain-containing protein; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulfate; MSP, methylation-specific PCR; ORF, open reading frame; EGF, epidermal growth factor; TGF- β , transforming growth factor β ; mRNA, messenger RNA.

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paring gene expression profiles of HCC with those of corresponding noncancerous liver through the generation of a large set of ESTs and cDNA microarray analysis.¹³ These data indicated that a number of known genes that related to oncogenesis, tumor suppression, and hepatic differentiation were deregulated in hepatocarcinogenesis, which was confirmed by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). Moreover, many deregulated novel genes were isolated, the role of which in hepatocarcinogenesis is under investigation. Among them, a liver-specific gene that encodes a novel zona pellucida (ZP) domain-containing protein, named LZP (for liver-specific ZP domain-containing protein), was isolated and characterized in this study.

Materials and Methods

Rapid Amplification of cDNA Ends. For 5'–rapid amplification of cDNA ends (RACE) of human LZP cDNA, primers were designed by reference to the sequence of our clone GLCAVE06 (GenBank accession AV647176). Primer sequences are hGSP, CTCTCCACCAAGCTTCCAAGCCGCT; hNGSP, CGTGGAACTCGCAGGTCACCGG. For mouse LZP cDNA, we performed 5' and 3'–RACE PCR according to the sequence of one mouse EST, EF-9.¹⁴ The primer sequences are mGSP1, GCCGCAGGTCTTGAGTGAGAAGACGATG; mNGSP1, GACGTGGGTGCCATTGGACATCCTC; mGSP2, AGCGACGGGAAGACTTGCGAAGACATC; mNGSP2, TGTGCAAGTCCAGCGCATTGAAGTG. Marathon-ready human and mouse liver cDNAs (Clontech, Palo Alto, CA) were used as a template, and the PCR reaction was performed according to the manufacturer's protocol.

Extraction of RNA and RT-PCR. Total RNA was extracted with TRIZOL reagent (Life Technologies Inc., Gaithersburg, MD) according to the manufacturer's protocol. Reverse transcription was carried out at 42°C for 1 hour in a 20- μ L reaction mixture that contained 1 μ g of total RNA, 10 pmol of oligo-dT, and 200 units of SuperScript II reverse transcriptase (Life Technologies Inc.). Human LZP and β -actin as internal control were coamplified in 1 PCR reaction with 0.5 μ L of RT product as template. Sequences of primers are hLZP forward, CTGAGGATAACCACACTTGCCAAGTC; hLZP reverse, CGAGGTTGCTGGCCACAATCTT; β -actin forward, TCACCCACACTGTGCCCATCTACGA; β -actin reverse, CAGCGGAACCGCTCATTGCCAATGG. The PCR reaction was performed as follows: initial 5 minutes at 94°C and then 33 cycles of 30 seconds at 94°C; 30 seconds at 52°C, 30 seconds at 72°C, and a final 7 minutes at 72°C.

The PCR products were separated by electrophoresis on a 2% agarose gel.

Northern Blot Analysis. A 15- μ g portion of total RNA 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-linking. Partial cDNA of hLZP (1-1,400 nt) and mLZP (26-1,600 nt) were labeled with [α -³²P]dCTP with Random Primer DNA Labeling Kit (TaKaRa Inc., DaLian, China). Homemade nylon membrane and Human Multiple Tissue Northern Blot (Clontech) were hybridized according to the manufacturer's protocol. After hybridization, the membranes were washed in 2 \times sodium saline citrate, 0.05% sodium dodecyl sulfate (SDS) for 30 minutes at room temperature and then in 0.1 \times sodium saline citrate, 0.1% SDS for 30 minutes at 50°C and visualized by autoradiography.

Cell Transfection and Immunofluorescence Microscopy. The expression plasmid pcDNA3.1-hLZP-Myc was transfected transiently into human liver cell line L02 on polylysine-treated slides with LipofectAMINE reagents (Life Technologies, Inc.). After incubation at 37°C for 60 hours, the fixed cells were blocked with bovine serum albumin and stained with anti-Myc monoclonal antibody (Clontech) or anti-hLZP polyclonal antibody overnight at 4°C, followed by incubation with fluorescein isothiocyanate–conjugated anti-mouse immunoglobulin G antibody (Gibco BRL, Grand Island, NY) at 4°C for 2 hours. After rinsing, the slides were analyzed with a microscope.

Selection of Stable Transfected Cells. HCC cell line SMMC-7721 and liver cell line L02 were transfected with pcDNA3.1-LZP (with or without cMyc tag) and empty pcDNA3.1 as control with LipofectAMINE reagents (Life Technologies, Inc.). Sixty hours after transfection, G418 (Life Technologies, Inc.) was added to the medium at a final concentration of 700 μ g/mL (for SMMC-7721) or 500 μ g/mL (for L02). After 3 weeks, clones were picked individually and expanded. The expression of hLZP in each individual clone was checked by Western blot using anti-LZP polyclonal antibody.

Western Blot Analysis. Samples were separated by 10% SDS–polyacrylamide gel electrophoresis, followed by transfer to Hybrid-P polyvinyl difluoride membrane (Amersham Pharmacia Biotech, Ltd.). After blocking in phosphate-buffered saline containing 5% bovine serum albumin and 0.1% Tween-20, the membrane was incubated with anti-Myc monoclonal antibody (Clontech) or anti-LZP polyclonal antibody at room temperature for 2 hours, followed by incubation with a horseradish peroxi-

dase-linked secondary antibody (Gibco BRL) at room temperature for 2 hours. The signals were detected using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Ltd.).

In Situ Hybridization. The frozen human liver tissues were sectioned with a cryostat at 14 μm and thawed onto Probe On slides (Fisher Scientific, Pittsburgh, PA). The oligonucleotide probe against *hLZP* (¹⁴⁴⁰CCCGGATGAGGTAGTATTTTCAGGACCTCGTCGATCTTGGA¹⁴⁰¹) was labeled at the 3'-end by [α -³⁵S]dATP using terminal deoxynucleotidyl transferase (Amersham Pharmacia Biotech, Inc.) according to the manufacturer's protocol. The sections were hybridized for 16 to 18 hours at 42°C. Then the slides were dipped in 50% NTB2 nuclear track emulsion (Amersham Pharmacia Biotech, Inc.), exposed in the dark at -20°C for 4 to 8 weeks, and developed in D-19 for 3 minutes.

Purification of Recombinant Protein and Production of Polyclonal Antibody. *Escherichia coli* strain BL21 was transformed with pGEX-LZP-(26-250) and grown at 37°C in 2 \times YT medium containing 100 $\mu\text{g}/\text{mL}$ ampicillin to reach $A_{600} = 0.6$. After additional 5 hours at 28°C with isopropylthio- β -D-galactoside at a concentration of 300 $\mu\text{mol}/\text{L}$, cells were harvested and suspended in 1 \times phosphate-buffered saline containing 5 mmol/L dithiothreitol and 100 $\mu\text{g}/\text{mL}$ phenylmethylsulfonyl fluoride. After sonication, the glutathione-S-transferase (GST)-LZP fusion protein was purified using GST affinity columns (Amersham Pharmacia Biotech, AB, Uppsala, Sweden) according to the manufacturer's protocol. Then the GST portion was removed by digestion with Thrombin (Sigma, St. Louis, MO) at a concentration of 2.5 U/mL for 2 hours at room temperature. Three rabbits were immunized 3 times with standard procedures using 2 mg of LZP (amino acids 26-250) as antigen for injection. The antiserum then was purified using Protein G Sepharose 4 Fast Flow (Amersham Pharmacia Biotech, AB) according to the manufacturer's protocol.

Immunocytochemistry. 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 minutes followed by incubation with 3% H₂O₂ in methanol at 37°C for 30 minutes to quench endogenous peroxidase activity. After blocking in 20% goat serum in phosphate-buffered saline at 37°C for 30 minutes, the slides were incubated with anti-LZP polyclonal antibody at 37°C for 2 hours 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 hour. The signals were detected using

Diaminobenzidine Substrate Kit (Vector Laboratories, Inc. Burlingame, CA).

Analysis of Cell Growth, Cell Cycle, and Apoptosis. For cell growth analysis, 96-well plates were seeded with 5×10^2 cells (for SMMC-7721) or 1×10^3 cells (for L02) per well. Cell growth was determined every day by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) conversion using the CellTiter 96 Aqueous Nonradioactive Cell Proliferation Assay System (Promega Co., Madison, WI) according to the manufacturer's protocol. For cell cycle analysis, cells were harvested and fixed with 70% ethanol at -20°C for 24 hours, then resuspended cells were added 10 μL propidium iodide (BD Biosciences Pharmingen, San Diego, CA) followed by incubation for 20 minutes at room temperature, then analyzed on a FACSCalibur instrument (BD Biosciences Pharmingen). For cell apoptosis analysis, 1×10^6 cells were harvested with Non-enzymatic Cell Dissociation Solution (Sigma), then probed using Annexin V-FITC Apoptosis Detection Kit II (BD Biosciences Pharmingen) according to the manufacturer's protocol.

Bisulfite Treatment of DNA and Methylation-Specific PCR. The methylation status of the putative promoter of *hLZP* was analyzed by methylation-specific PCR (MSP) on the sodium bisulfite converted DNA.¹⁵ Briefly, 2 μg DNA from each sample was denatured by 0.2 mol/L NaOH at 37°C for 10 minutes, then incubated at 50°C for 16 hours after adding 30 μL of freshly prepared 10-mmol/L hydroquinone and 520 μL of freshly prepared 3-mol/L sodium bisulfite at pH 5.0. The modified DNA was purified using the Wizard DNA purification resin (Promega Co.) according to the manufacturer's protocol, followed by incubation with 0.3 mol/L NaOH for 5 minutes at room temperature. Finally, the DNA was ethanol precipitated and resuspended in 20 μL H₂O. PCR was performed in a volume of 15 μL with 1 μL of sodium bisulfite-treated DNA as template with HotStar Taq DNA polymerase (Qiagen, Hilden, German). After 5 minutes at 94°C for denaturation, 35 cycles of 30 seconds at 94°C, 30 seconds at 64°C for unmethylation-specific primers (U) or 66°C for methylation-specific primers (M), and 30 seconds at 72°C were carried out followed by a final 5 minutes at 72°C. The sequences of primers are U-forward, TGGGAGGTTAAGGTAGGTGGATTAT; U-reverse, ACAAACTTCACCTCCCAAATTCACA; M-forward, GGAGGTTAAGGTAGGCGGATTAC; M-reverse, CTTCGCCTCCCGAATTCACG. The PCR products were separated by 2% agarose gel electrophoresis and cloned into T-easy vector (Promega Co.) and sequenced.

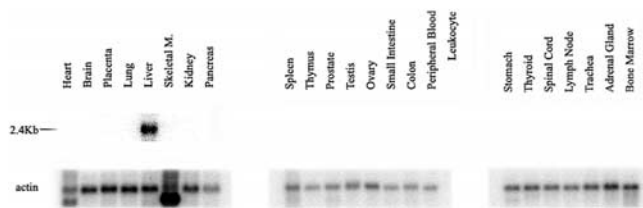


Fig. 2. Tissue expression pattern of *hLZP*. Human Multiple Tissue Northern blot membranes (Clontech) were hybridized with an *hLZP* cDNA probe. A single 2.3-kb band was detected only in liver. β -actin was used as control.

Results

Isolation and Characteristics of *LZP* cDNA. A large set of ESTs and cDNA microarray data derived from HBV-positive HCC and adjacent liver were analyzed by our group previously.¹³ Many deregulated novel genes/ESTs that associated possibly with malignant transformation or maintenance were encountered by comparing the data between HCC and adjacent liver. The data indicated that one EST, GLCAVE06 (GenBank accession no. AV647176), could be down-regulated in hepatocarcinogenesis because there were 12 copies in adjacent liver whereas there were only 2 copies in HCC, which is significantly different statistically ($P < .01$). Moreover, this human EST shares high similarity with a liver-specific mouse EST, EF-9.¹⁴ Then 5'-RACE was performed to isolate the full-length cDNA of this novel human gene based on sequence of the corresponding human EST clone, which contains a 1.4-kb insert with a poly-A tail but lacking initial ATG. Finally, the full-length cDNA of 2,255 bp, which coincides with Northern blot analysis and contains the complete open reading frame (ORF) encoding 545 amino acids with a calculated molecular mass of 60 kd was isolated (Figs. 1A and 2). Moreover, both *in vitro* translation and expression of *hLZP* in mammalian cells showed a nearly 60-kd band, which was consistent with theoretical molecular mass (data not shown). Similarly, 5'-RACE and 3'-RACE were performed to isolate its mouse counterpart according to the sequence of EF-9, which contains the partial ORF without start codon in 5'-end and stop codon in 3'-end. The results showed that mouse *LZP* has two isoforms, 2.2 kb and 2.7 kb, respectively, sharing the identical ORF, except that the long isoform has an additional 500-bp region in the 3'-untranslated region, which coincides with Northern blot analysis (Fig. 3, also see Fu and Kamps¹⁴). Sequence analysis revealed that different isoforms utilize distinct polyA signals AATAAA at position 2,157 or 2,647 in the 3' untranslated region, respectively. Mouse *LZP* contains an ORF of 1,638 bp that encodes 546 amino acids, which shares 89% identity and similar architecture with human *LZP* protein.

The amino acid sequence of *LZP* was analyzed using the Simple Modular Architecture Research Tool (SMART) database,¹⁶ which revealed that it has a signal peptide in its *N*-terminal region, followed by 3 epidermal growth factor (EGF)-like domains and then a ZP domain in the C-terminal region. *LZP* shares similar domain architecture with many known ZP domain-containing proteins, such as ZP2, ZP3, transforming growth factor β (TGF- β) receptor III, and uromodulin, in which the ZP domains are highly conserved (Fig. 1B and C). The phylogenetic analysis showed that *LZP* is closer to ZP3, endoglin, and TGF- β receptor III (Fig. 1D). The full-length cDNA of human *LZP* was compared with human genome database in GenBank by BlastN software. The result indicated that *hLZP* shares an identical DNA

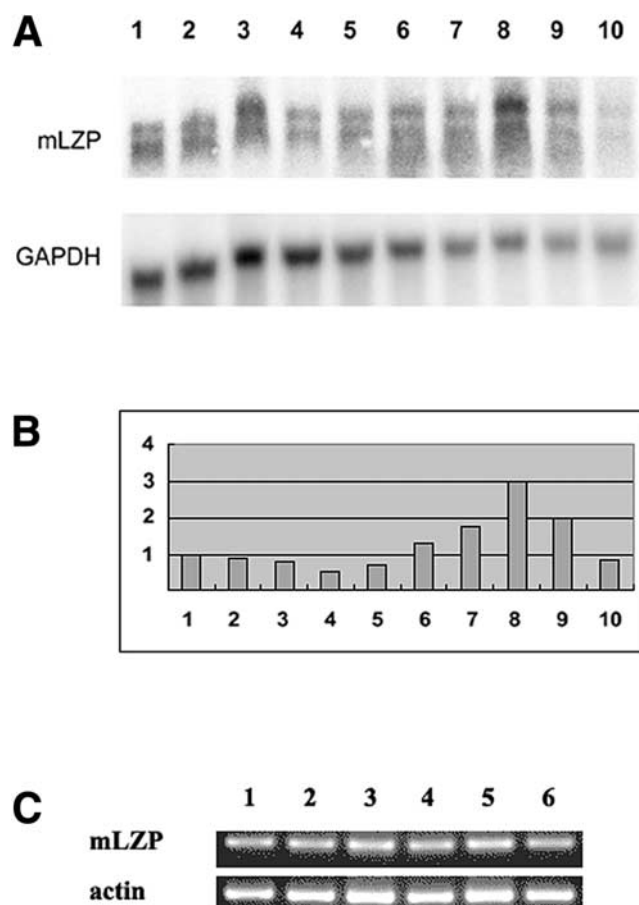


Fig. 3. Developmental expression pattern of *mLZP*. (A) The expression level of *mLZP* mRNA in mouse liver of different developmental stages was analyzed by Northern blot using part of the *mLZP* cDNA as a probe. The expression of mouse *GAPDH* mRNA was also shown as a control. Lane 1, E11.5 liver; lane 2, E13.5; lane 3, E15.5; lane 4, E17.5; lane 5, E19.5; lane 6, 1 day after birth; lane 7, 3 days after birth; lane 8, 7 days after birth; lane 9, 14 days after birth; lane 10, adult. (B) The relative expression level of *mLZP* mRNA was normalized by *GAPDH* level as shown in A. (C) The mRNA level of *mLZP* in early mouse embryo was analyzed by RT-PCR. β -actin also was shown as control. Lane 1, E6.5; lane 2, E7.5; lane 3, E8.5; lane 4, E9.5; lane 5, E10.5; lane 6, E 11.5.

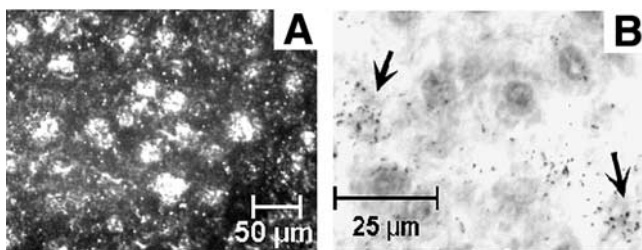


Fig. 4. *In situ* hybridization of *hLZP*. *In situ* hybridization analysis was performed on human liver tissue with an antisense oligonucleotide probe against *hLZP* mRNA. (A) Dark field view. (B) Bright field view (signal indicated with arrow). The signals are detected in hepatocytes.

sequence with clone RP11-522H2 localized on chromosome 10q21.3, in which the gene spans about 40 kb, containing 9 exons and 8 introns (Fig. 1E). Similarly, the mouse *LZP* gene also contains 9 exons and 8 introns, although it only spans about 20 kb harbored on mouse chromosome 10B4.

Hepatocyte-Specific Expression of *LZP*. To determine the tissue expression pattern of *hLZP*, Northern blot analysis was performed using Human Multiple Tissue Northern blot membrane (Clontech). The result indicated that a single band of about 2.3 kb was detected only in liver out of 23 tissues, which implied that *hLZP* is expressed specifically in liver (Fig. 2). The data matched that of similar analysis using mouse EF-9 as probe among mouse tissues.¹⁴ The tissue-specific expression of *LZP* implied that this gene could be involved in liver development. To find out the expression pattern of *LZP* during liver development, Northern blot was performed using *mLZP* as probe to detect mouse liver of different developmental stages. The result showed that expression of *mLZP* starts in early fetal liver (at least from E11.5) (Fig. 3A). Moreover, the expression level changes in different developmental stages. The expression decreases to a minimal level at E17.5, then increases gradually to the maximal level at about 7 days after birth. The expression of *mLZP* in adult liver stabilizes at a little lower level than that of E11.5 (Fig. 3B). There is no visible difference between the expression levels of the 2 isoforms. To study whether the expression was initiated before liver onset, total RNA from earlier mouse embryo was used as template for RT-PCR to detect the expression of *mLZP*. The result revealed that *mLZP* was expressed very early, at least from E6.5 (Fig. 3C).

It is known that liver contains many types of cells, in particular two types of tissue-specific cell: hepatocyte and bile duct cell. To find out which kind of cell *LZP* is expressed in, *in situ* hybridization and immunocytochemistry on human liver sample were performed to detect messenger RNA (mRNA) and protein of *hLZP*, respectively. The results indicated that *hLZP* is expressed only in hepatocytes (Figs. 4 and 5).

Subcellular Localization of *hLZP* on Nuclear Membrane.

To determine the subcellular localization of *hLZP*, two methods, indirect immunofluorescence for tagged *hLZP* and immunocytochemistry for endogenous *hLZP* protein, were used to define the issue. Myc-tagged *hLZP* expression plasmid was transfected into human liver cell line L02. Indirect immunofluorescence assay with anti-myc antibody showed that myc-tagged *hLZP* was localized clearly on nuclear membrane as ring cycle, although minor protein in cytoplasm was labeled (Fig. 6A). Furthermore, immunocytochemistry assay on human liver samples indicated that the endogenous *hLZP* protein also was detected on nuclear membrane by anti-*hLZP* antibody (Fig. 5). However, *LZP* was predicted originally as a potential secretory protein by bioinformatics tools because it has a deduced signal peptide. Moreover, most known *ZP* domain-containing proteins were confirmed as secretory proteins or cell membrane proteins. To define whether *LZP* protein was secreted out from cells, plasmid pcDNA3.1-*hLZP*, without any tag, was transfected into human liver cell L02. The *hLZP* protein was detected both in cell lysates and culture medium by Western blot with anti-*LZP* antibody. Interestingly, *hLZP* protein also was found in plasma from healthy humans (Fig. 6B), whereas it was not detected in human bile. The secretory *hLZP* is a little bigger than intracellular *LZP*, which suggested that the protein might be glycosylated in the secretory process because there are several *N*-glycosylation sites in *LZP* based on bioinformatics analysis. Those data revealed that *hLZP* protein could be secreted out from hepatocytes as secretory protein, although the protein is localized mainly on the nuclear envelope.

Lower Expression of *hLZP* in HCC. Our previous EST data implied that the *hLZP* gene could be down-regulated in hepatocarcinogenesis because there were 12 copies in adjacent liver but only 2 copies in HCC. To verify the hypothesis, Northern blot analysis was performed using 5 pairs of samples, cancerous and adjacent noncancerous liver, from patients with HCC. The result showed that the hybridized band of *hLZP* disappeared completely or weakened significantly in all 5 cancerous tissues (Fig. 7A). To strengthen the evidence, semiquantitative RT-PCR was used to test additional 26 pairs of samples from patients with HCC. The result indicated that *hLZP* could not be detected or decreased dramatically in cancerous tissue. Simultaneously, *hLZP* was not detected in some HCC cell lines such as 7404, 7721, HepG2, and human liver cell line L02 (Fig. 7B). These integrated data from 31 patients, which showed that *hLZP* was not detected in 25 of 31 (80.6%) HCC tissues and decreased significantly in 6 of 31 (19.4%) HCC tis-

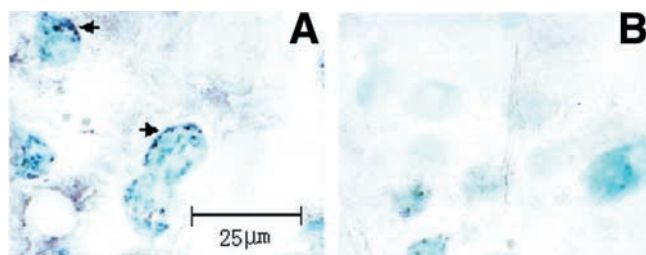


Fig. 5. Immunocytochemistry of hLZP. Immunocytochemistry was performed on human liver with anti-LZP polyclonal antibody. The nucleus was stained with methyl green. The positive signals are indicated with arrows. (A) Adjacent noncancerous liver. (B) Cancerous liver. The signals are detected only on nuclear membrane of hepatocytes from noncancerous liver.

sues, revealed that the transcriptional expression of *hLZP* could be weakened or even ceased during hepatocarcinogenesis. Immunocytochemistry was performed with anti-hLZP polyclonal antibody to examine the protein level of hLZP in some pathologic slides from HCC patients. The hLZP protein was not detected in these slides, which confirmed the conclusion (Fig. 5).

Methylation Profile of Putative *hLZP* Promoter in HCC. To investigate the possible mechanism of decreased transcriptional activity of *hLZP* in HCC samples, MSP for detecting the methylation status of *hLZP* promoter was performed. There is a CpG-rich region in the putative *hLZP* promoter, and specific primers for MSP were designed based on the sequence of this region (Fig.

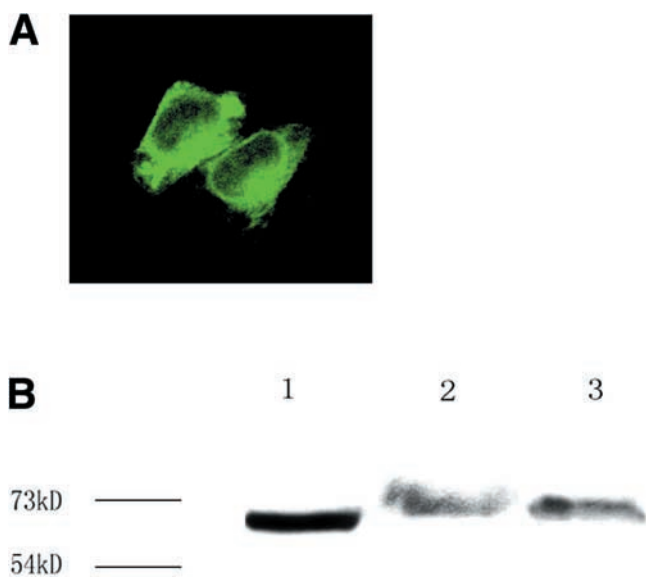


Fig. 6. Subcellular localization of hLZP. (A) L02 cells were transfected with pcDNA3.1-hLZP-Myc. After 60 hours, the cells were fixed and stained with anti-Myc monoclonal antibody. The fluorescence is mostly detected on nuclear membrane. (B) hLZP was detected by Western blot. Lane 1, cell lysates of L02 cells transfected with pcDNA3.1-hLZP; lane 2, culture medium of L02 cells transfected with pcDNA3.1-hLZP; lane 3, human plasma.

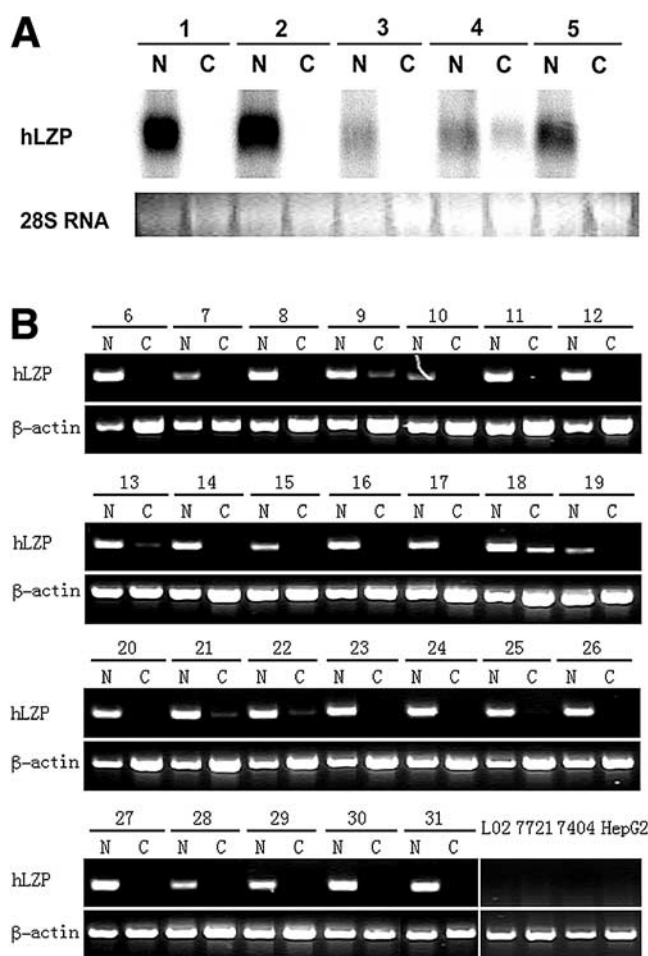


Fig. 7. Transcriptional expression of *hLZP* mRNA in HCC and adjacent liver samples. (A) The expression level of *hLZP* mRNA in human HCC and adjacent liver samples was analyzed by Northern blot. The 28S rRNA was also shown as a control. C, cancerous liver tissue; N, corresponding adjacent noncancerous liver tissue. (B) RT-PCR was performed to analyze the *hLZP* mRNA expression in paired HCC/adjacent livers and some liver cell lines. L02 is a human liver cell line while 7721, 7404, and HepG2 are HCC cell lines. β -actin was used as control.

8A). The results revealed that the methylation and unmethylation statuses occur simultaneously in human normal livers and non-HCC samples, implying possible genomic imprinting in the putative *hLZP* promoter. However, the level of methylation in 5 HCC samples (D1-D5) among 8 pairs of samples examined was elevated dramatically, whereas unmethylated PCR products were detected rarely in those samples (Fig. 8B), although both methylated and unmethylated PCR products were generated in three other cancerous tissues (D6-D8). The MSP results were confirmed by sequencing the PCR products (Fig. 8C). These data implied that the decreased transcriptional activity of *hLZP* in HCC samples, at least in a part of patients with HCC, could be associated with the methylation status of the putative *hLZP* promoter.

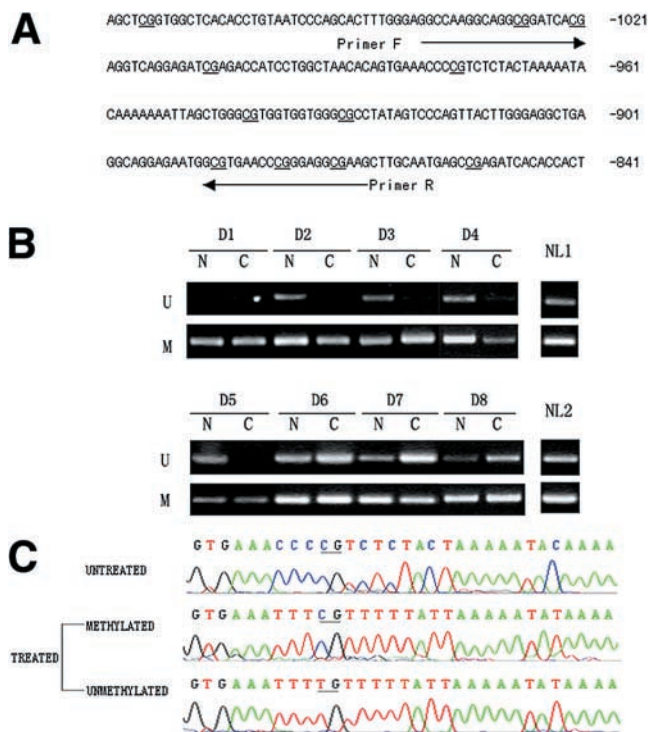


Fig. 8. Methylation profile of the putative *hLZP* promoter in human normal liver and HCC samples. (A) The CpG-rich region of the putative *hLZP* promoter. The CpG dinucleotides are indicated by **underlines**. Primers F and R indicate the sequences that were used for designing the methylation-specific primers. (B) Methylation status of *hLZP* was examined by MSP assay. The electrophoretic patterns of PCR products were presented. C, cancerous liver tissue; N, corresponding adjacent noncancerous liver tissue; NL, normal liver tissue; U, amplified by the unmethylation-specific primers; M, amplified by the methylation-specific primers. (C) The DNA sequences of representative PCR products by automatic DNA sequencer. The CpG dinucleotides are indicated by **underlines**.

Effect of LZP on Cell Growth. To address the functions of LZP, human HCC cell line SMMC-7721 and liver cell line L02 with *hLZP* protein expression were established by stable cell transfection. The *hLZP* protein was constitutively expressed in the selected 5 clones, 2 from L02 cells and 3 from SMMC-7721 cells, with different expression levels (Fig. 9A). However, the selected cell lines with *hLZP* protein did not exhibit unusual cellular biologic behaviors examined, including cell growth, cell cycle, and apoptosis. The studies on cell growth by MTS conversion revealed that the growth rate of the cells with *hLZP* protein, whether L02 or SMMC-7721, is similar to that of the cells without *hLZP* protein during 7 culture days (Fig. 9B), suggesting that *hLZP* protein could not inhibit cell proliferation. Moreover, cell cycle and apoptosis of those recombinant cell lines, examined by FACS, appeared to be similar to that of the cells as mock or control, implying that *hLZP* protein could have no significant effect on cell cycle and cell apoptosis (data not shown).

Discussion

The ZP domain, which contains about 260 amino acid residues, has been recognized in a number of receptor-like eukaryotic glycoproteins.¹⁸ This domain was considered an extracellular domain, followed by either a transmembrane region or a glycosyl phosphatidylinositol anchor in the C-terminal region. It contains 8 cysteines probably involved in disulfide bonds. There was evidence indicating that the ZP domain is responsible for polymerization of these proteins into filaments of similar supramolecular structure.¹⁹ In the SMART database, 238 putative ZP domain-containing proteins were found only in metazoan and none in yeast and plants, which suggested that the ZP domain-containing proteins came into being in a rather late stage during evolution. In human beings, several ZP domain-containing proteins, such as ZP2, ZP3, TGF- β receptor III, Hesin, uromodulin, tectorin, etc., were recognized before. In this report, LZP was identified as a novel member of the ZP protein family. Like other ZP domain-containing proteins, LZP has a putative signal peptide and a large ZP domain. Moreover, LZP also has three additional EGF-like domains upstream of the ZP domain, so it has similar domain architecture with uromodulin, except for the different position of EGF-like domains. Except for certain fish homologs of ZP2/ZP3 proteins, most known ZP domain-containing proteins have a transmembrane domain or a glycosyl phosphati-

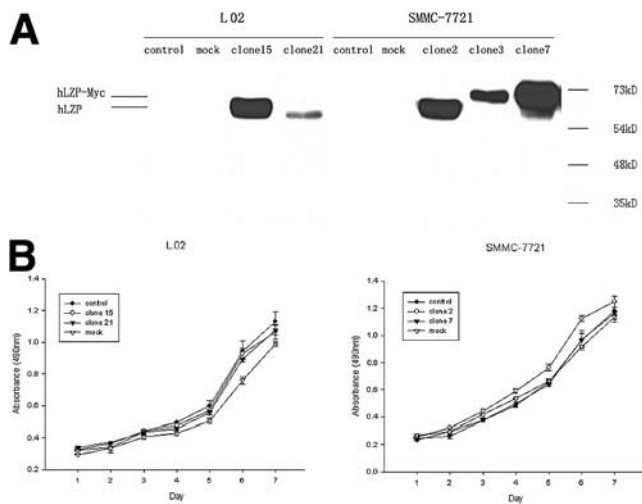


Fig. 9. The effect of *hLZP* on cell proliferation of human cell lines L02 and SMMC-7721. (A) Cell clones with *hLZP* protein by stable cell transfection were identified by Western blot assay. Control, transfected with empty vector pcDNA3.1; mock, transfected with plasmid containing *hLZP* cDNA, but not expressed. Clone number 15 and 21 from L02, and clone number 2 from SMMC-7721 express *hLZP* protein without tag; clones 3 and 7 express *hLZP* protein with cMyc tag, as indicated. (B) Cell growth curves of the recombinant cells with or without *hLZP* were analyzed by MTS conversion. Each sample was tested in triplicate and error bars are included.

dylinositol anchor in the C-terminal region and were located on the cell membrane, some of which can even be secreted out of cells after digestion with proteases. However, like ZP2/ZP3 homologs of some fish, LZP has no transmembrane domain or a glycosyl phosphatidylinositol anchor downstream of the ZP domain. Moreover, our data revealed that hLZP protein could be secreted out of cells, albeit it was localized mainly on the nuclear membrane. The ZP2/ZP3 homologues of certain fish are synthesized by the liver and travel in the bloodstream to their assembly site around eggs.^{20,21} Using a monoclonal antibody against chorion proteins of the sea bass *Dicentrarchus labrax*, researchers detected signals in hepatocytes, but the signal did not reveal evident subcellular localization patterns.²²

The present evidence suggested that ZP domain-containing proteins are involved in many important biological processes. ZP2 and ZP3, which are involved in sperm adhesion to the zona pellucida, are indispensable in acrosome reaction. ZP3 first binds to specific sperm proteins, mediating sperm contacts with the oocyte and then ZP2 acts as a second sperm receptor reinforcing the interactions.^{23,24} TGF- β receptor III (betaglycan) is thought to be involved in capturing and retaining TGF- β for presentation to the type II receptor, and endoglin, another ZP domain-containing protein, can increase the binding of TGF- β to type I and II receptors both.²⁵⁻²⁷ Uromodulin, also named Tamm-Horsfall glycoprotein, was considered as the receptor of interleukin 1 α , interleukin 1 β and tumor necrosis factor, and can suppress antigen-specific T-cell proliferation *in vitro*.^{28,29} GP-2 is the major component of pancreatic secretory granule membranes and is suggested to play an important role in endocytosis along with src kinases and caveolin.³⁰⁻³² Tectorin α and β are presumed to play a key role during mouse inner ear development.³³ Hesin, encoded by DMBT1, which is frequently deleted in medulloblastoma and glioblastoma, is implicated in epithelial terminal differentiation.^{34,35} Our data revealed that LZP is expressed in an early embryo developmental stage, at least from day 6.5 of mouse gestation (E6.5) before mouse liver onset by E8.5,³⁶ suggesting that it could be involved in liver development and differentiation.

Interestingly, the mRNA level of *hLZP* decreased dramatically, even disappeared in all 31 HCC patients and 4 liver cell lines that we examined. Similar to some evidence indicating that methylation can contribute to the inactivation of tumor suppressor genes in hepatocarcinogenesis,^{37,38} the methylation status of putative *hLZP* promoter could induce the decreased transcriptional activity of the gene. However, our data did not suggest that the loss of hLZP might play an important role in hepato-

carcinogenesis because overexpression of hLZP in some liver cell lines did not inhibit cell proliferation, influence cell cycle, or induce apoptosis. So it seems the down-regulation of *hLZP* could be a consequence, not a cause, of the dedifferentiation status of HCC. Notwithstanding, the fact that hLZP cannot be detected in the majority of HCC samples by Northern blot, RT-PCR, and immunocytochemistry suggested that it could be used as potential negative biomarker, combining with α -fetoprotein as positive biomarker for HCC diagnosis.

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