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The antigen for Hep Par 1 antibody is the urea cycle enzyme carbamoyl phosphate synthetase 1

Samantha L Butler*, Huijia Dong*, Diana Cardona, Minghong Jia, Ran Zheng, Haizhen Zhu, James M Crawford and Chen Liu

Hepatocyte paraffin 1 (Hep Par 1), a murine monoclonal antibody, is widely used in surgical pathology practice to determine the hepatocellular origin of neoplasms. However, identity of the antigen for Hep Par 1 is unknown. The aim of this study was to characterize the Hep Par 1 antigen. To identify the antigen, immunoprecipitation was used to isolate the protein from human liver tissue, and a distinct protein band was detected at approximately 165 kDa. The protein band was also present in small intestinal tissue, but was not present in several other non-liver tissues nor in three human hepatocellular carcinoma cell lines, Huh-7, HepG2, and LH86. The protein was purified and analyzed by mass spectrometry. It was identified as carbamoyl phosphate synthetase 1 (CPS1). CPS1 is a rate-limiting enzyme in urea cycle and is located in mitochondria. We demonstrated that hepatoid tumors (gastric and yolk sac) were immunoreactive with both Hep Par 1 antibody and anti-CPS1 antibody, further confirming the results of mass spectrometric analysis. We found that the three human hepatocellular carcinoma cell lines do not express either CPS1 RNA or protein. We confirmed that the gene was present in these cell lines, suggesting that suppression of CPS1 expression occurs at the transcriptional level. This finding may have relevance to liver carcinogenesis, since poorly differentiated hepatocellular carcinomas exhibit poor to absent immunoreactivity to Hep Par 1. In conclusion, we have identified the antigen for Hep Par 1 antibody as a urea cycle enzyme CPS1. Our results should encourage further investigation of potential role that CPS1 expression plays in liver pathobiology and carcinogenesis.

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The histological distinction between hepatocellular carcinomas (HCC) and metastatic adenocarcinoma to the liver can sometimes be a challenging dilemma for surgical pathologists, particularly given the histological variants of HCC that can occur. In addition, tumors in other sites can display hepatoid morphologic features, adding to the diagnostic challenge when considering their metastasis to the liver. In the end, a wide panel of immunohistochemical markers is often used for the differential diagnosis of HCC, cholangiocarcinoma and metastatic adenocarcinoma. These markers include alpha-fetoprotein (AFP), polyclonal carcinoembryonic antigen (pCEA), and alpha-1-antitrypsin.[1] None of these markers, however, are highly specific or sensitive for hepatocyte differentiation.

In 1993, Wennerberg *et al*,[2] developed a new monoclonal antibody specific for hepatocytes in formalin-fixed, paraffin-embedded tissues. This antibody, named hepatocyte paraffin 1 (Hep Par 1, clone OCH1E5.2.10), was generated using tissue extracts from a formalin-fixed failed allograft liver. Subsequent studies showed a high sensitivity and specificity of Hep Par 1 for normal hepatocytes and neoplastic hepatic tissue.[3–6] Additional recent studies examined Hep Par 1 reactivity in a variety of non-hepatic tissues, both benign and neoplastic.[7–10] A large number of gastric adenocarcinomas show Hep Par 1 reactivity.[11] Rare examples of cholangiocarcinomas, yolk sac tumor, and adenocarcinomas of the ovary, adrenal cortex, lung, endocervix, colon, and pancreas have shown focal Hep Par 1 staining.[9,12,13] Hep Par 1 reactivity is also seen in benign small intestinal mucosa and intestinal metaplasia of the esophagus and stomach.[14]

Although the Hep Par 1 antibody exhibits excellent sensitivity and specificity for well-differentiated hepatocytes,

Department of Pathology, Immunology and Laboratory Medicine, University of Florida, Gainesville, FL, USA
Correspondence: Dr C Liu, MD, PhD, Department of Pathology, Immunology and Laboratory Medicine, University of Florida, 1600 SW Archer Rd, PO Box 100275, Gainesville, Florida 32610, USA. E-mail: Liu@pathology.ufl.edu
*These authors contributed equally to this work.

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the identity of the antigen and its role in liver biology and carcinogenesis are still unknown, constituting one of the great enigmas of diagnostic pathology. Establishing the identity of the antigen is critical, for a number of reasons. First, by knowing the identity of this antigen, additional antibodies of potentially greater diagnostic utility may then be generated. Second, this antigen is predominantly liver-specific. Examining expression patterns for this protein in normal and cancerous liver tissues will likely benefit studies on liver cell biology and pathobiology. Third, such identification may be of substantial value in understanding the significance of the Hep Par 1 reactivity in a number of non-liver cancers, particularly since such expression is associated with a 'hepatoid' cellular phenotype.

Herein we report the identification and characterization of the Hep Par 1 antigen. Through the use of immunoprecipitation combined with mass spectrometry, this antigen is identified as carbamoyl phosphate synthetase 1 (CPS1), a relatively liver-specific, intramitochondrial rate-limiting enzyme in the urea cycle. Moreover, our data show that CPS1 is not expressed in human liver cancer cell lines in cell culture, raising the question of whether suppression of CPS1 expression has relevance to liver carcinogenesis.

MATERIALS AND METHODS

Tissue Samples

A total of five liver tissue samples, obtained from the University of Florida Shands Hospital, were used to establish the identity of the Hep Par 1 antigen. These tissue samples include normal liver resected for metastatic colon cancer (three patients, two males and female) and primary hepatocellular carcinoma tissue (two patients, males). None of the liver tissues were positive for any known viral infections. Normal non-liver tissue samples from the surgical bench were used to determine the liver specificity of the Hep Par 1 antigen. The normal tissue collected included: skin, thyroid, small intestine, appendix, gallbladder, kidney, and spleen. The surgical pathology case files at the University of Florida from 2000 to 2007 were searched to identify non-liver tumors with 'hepatoid' differentiation (cohesive polygonal tumor cells with abundant eosinophilic cytoplasm). One gastric carcinoma was identified with focal 'hepatoid' histological features. Three yolk sac tumors also were identified, one of which exhibited focal 'hepatoid' differentiation. Paraffin blocks were sectioned for the purposes of CPS1 and Hep Par 1 immunohistochemistry.

A Chinese hamster cell line (CHO) was used as a negative control for immunological reactivity to Hep Par 1. Protein extract also was prepared from three hepatocellular carcinoma cell lines, Huh-7, HepG2, and LH86.[15] Tissues and cell line homogenates were homogenized in protein lysis buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-CL pH 8.0, 2 mM DTT, 40 μg/ml PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin) followed by centrifugation (13 000 r.p.m., 8000 *g*) at 4°C for 10 min. Protein concentrations were determined from the supernatants using the BioRad Lowry Protein Assay and protein spectrophotometer (750 nm) as previously reported.[16]

Immunohistochemistry Staining

Immunohistochemistry, using Hep Par 1 antibody (obtained from DAKO, Carpinteria, CA) was performed on frozen liver tissue sections to determine if antigen detection was possible on non-formalin-fixed tissue. Sections (5 μm thick) were obtained in a freezing microtome and fixed in ethanol without any formalin exposure. Alternatively, formalin fixation of tissue, paraffin embedding, and sectioning (5 μm thick) were performed by routine methodology using antigen retrieval. Immunohistochemical reactions with Hep Par 1 were as reported previously.[10] The antibody dilution is 1:50. Polyclonal anti-CPS1 antibody was purchased from Abcam (Cambridge, UK).

Immunoprecipitation

Immunoprecipitation was used to purify the Hep Par 1 antigen from the liver tissue samples. For each sample, 60 μl (100 μg protein) of liver protein extract was added to a solution combination of protein lysis buffer as described above, and fresh proteinase inhibitor (40 μg/ml PMSF, 2 mM DTT, 2 μg/ml Aprotinin, 2 μg/ml Leupeptin) to a final volume of 500 μl. The resulting tissue extract solution was incubated with 7.5 μg of Hep Par 1 monoclonal antibody (135 mg/l concentration) overnight on a 4°C rocker. After overnight incubation, 50 μl of protein G agarose/sehparose bead slurry (Sigma, St Louis, MO, USA) was added to the antibody/tissue extract solution and incubated at 4°C for 2 h on a tube rocker. The beads were collected by centrifugation at 13 000 r.p.m. at 4°C for 5 min. The agarose beads were washed three times with 500 μl of cold 1 × TBS buffer with 0.1% Tween 20. The beads were resuspended in 20 μl of 2 × SDS sample buffer, boiled for 5 min, and collected by centrifugation. The resulting supernatant was analyzed by SDS-polyacrylamide gels (SDS-PAGE) as previously reported.[17]

Western Blot Analysis

Approximately 40 μg of total protein extracts from the liver tissue or non-liver tissues were loaded on 10% SDS-polyacrylamide gels. The gels were run at 100 V for approximately 1 h. The gels were electrophoretically transferred to polyvinylidene difluoride membrane (PVDF) (BIO-RAD, Hercules, CA, USA). The membranes were incubated overnight at 4°C in blocking buffer (1 × TBS containing 0.1% Tween 20 and 5% fat-free milk power (wt/vol)). Hep Par 1 monoclonal antibody (1:500 dilution) was added to the liver extract membrane and the non-liver tissue membranes in 1% bovine serum albumin (BSA) in 1% TBS containing 0.1% Tween 20 for 1 h on a rocker. Anti-actin antibody (1:1000 dilution) was used for an internal control. The membranes were washed three times for 10 min each with TBS containing 0.1% Tween 20, followed by incubation with goat anti-mouse

IgG-HRP from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) (dilution 1:2000) in TBS containing 0.1% Tween 2 0 and 5% fat-free milk for 1 h. After washing for three times as described above, the membrane was incubated with Supersignal West Pico Chemilunescent Substrate (Pierce Biotechnology, Rockford, IL, USA) solution for signal detection.

Mass Spectrometry

Gel pieces excised from the SDS-PAGE gels were washed twice with 50 μl of destain solution (50% v/v acetonitrile, 25 mM NH_4HCO_3) for 45 min to remove the Coomassie blue stain. The pieces were dried for 20 min. In-gel tryptic digestions were carried out by adding 15 μl, 5.0 mM NH_4HCO_3 containing 12.5 ng/ml trypsin and incubating at 37°C for 24 h. Peptides were extracted by incubation of the gel pieces with 10 μl of 50% v/v acetonitrile in water containing 0.1% of trifluoroacetic acid for 15 min. The extracted tryptic digest peptides were analyzed by liquid chromatography–electrospray tandem mass spectrometry (LC/MSMS). The liquid chromatographic separation of the peptide digests was run on a capillary C18 column with dual mobile phases of a gradient of ACN in water containing 0.1% HAc, and the mass spectrometric identification data were acquired using an LCQ deca mass spectrometer (Thermo Finnigan) performed in positive electrospray and data-dependent mode. The MSMS data for the digest sample from the distinct gel band were searched using MASCOT ion MSMS search against the NCBI_nr database with peptide tolerance of 2.0 Da and MS/MS tolerance of 0.8 Da. One missed cleavage for the trypsin digestion was allowed in the database search.

Cell Culture, RNA Extraction, RT-PCR, and PCR Analysis

All the three hepatocellular cell lines were cultured in DMEM medium with 10% fetal calf serum, as described before.[18] Primary human hepatocytes were obtained and cultured according to published protocol.[19] Procedures of RNA and DNA extraction from liver cancer cell lines, PCR, and RT-PCR analysis were previously published.[16] The primers for CPS1 RT-PCR analysis were: 5′ATGCGGCCGCAGCCACA AATCATCTTCAAAA; 5′ CGTCTAGATGAGAAAGTTGTGA ATCAGTTCC, and primers for PCR analysis were 5′-CAA CTCAGCATGGCATTGAC; 5′-ACAGCTGTCCTCCGAATCAC.

RESULTS

Hep Par 1 Antibody Detects a 165 kDa Protein in Liver Tissue

Hep Par 1 antibody was originally derived using formalin-fixed liver tissue.[2] Immunostains using this antibody in pathology practice are almost exclusively performed on formalin-fixed and paraffin-embedded tissue sections. It is unknown whether the antigen detection needs formalin fixation. Identification of the protein using formalin-fixed tissue would be extremely difficult due to protein cross-linking following formalin exposure. To answer this question, we performed immunohistochemical staining using Hep Par1 antibody on fresh frozen liver tissue sections, post-fixed with ethanol without any formalin exposure. As shown in Figure 1, Hep Par 1 reacted with the antigen in the fresh frozen liver tissue, as demonstrated by granular staining similar to formalin-fixed liver tissue section. This suggests

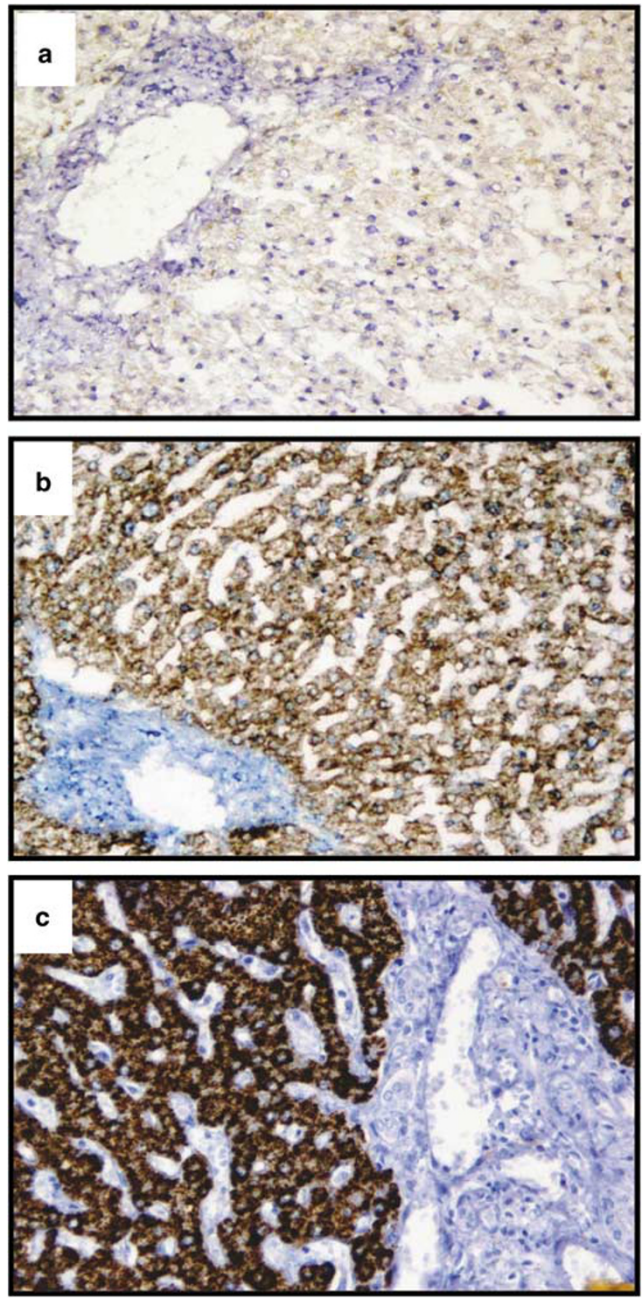


Figure 1 Immunohistochemistry of liver tissue with Hep Par 1 antibody. Normal liver tissue was used for immunoperoxidase staining with the Hep Par 1 antibody (Dako Cat#: M-7158, 1:50 dilution). (**a**) Negative control for Hep Par 1 immunostain, on fresh frozen liver tissue fixed with ethanol. (**b**) Hep Par 1 antibody immunostain of fresh frozen liver fixed with ethanol. (**c**) Hep Par 1 antibody immunostain in formalin-fixed liver tissue. Original magnification × 200.

that Hep Par 1 antigen is not formalin fixation or paraffin-induced, although its name connotes paraffin (hence the abbreviation 'Hep Par 1'). This finding justified use of fresh liver tissue to identify this antigen.

We next prepared protein extracts from fresh normal liver tissue, a human hepatocellular carcinoma cell line, Huh-7 as a potential positive, and the CHO cell line as a presumed negative. The protein extracts were resolved by SDS-PAGE followed by western blot analysis using the Hep Par 1 antibody. As shown in Figure 2, an abundant and specific 165 kDa band is detected only in the fresh liver extracts, but not in the Huh7 or CHO cell lines. This protein was designated as p165. The Huh7 cell line represents a poorly differentiated cancer cell line. Apparently, p165 is not present in this cell line.

Hep Par 1 Antigen is Present in Liver and Small Intestine Epithelium

The tissue distribution of p165 was next examined using western blot analysis. We prepared protein extracts from normal gallbladder, thyroid, small intestine, spleen, kidney, and skin. These protein extracts were analyzed by western blot analysis with Hep Par 1 antibody. The result revealed a distinct p165 band in the small intestine tissue, but not in other non-liver tissues examined (Figure 3). We then stained a section of normal intestinal tissue using Hep Par 1 antibody. As shown in Figure 3b, strong immunohistochemical reaction is present in the epithelial cells of the small intestine, similar to what has been reported before.[20]

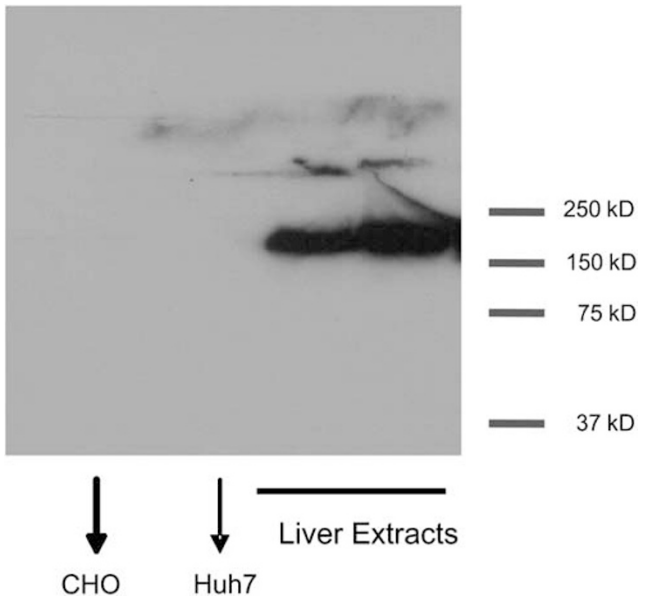


Figure 2 Western blot analysis using Hep Par 1 antibody. Protein extracts were made from normal liver tissue, Huh7, and CHO cell lines. Equal amounts of protein extracts were resolved in 10% SDS-PAGE, followed by western blot analysis using Hep Par 1 antibody (1:500 dilution). The antibody detected a 165 kDa band only in the liver tissue extracts (two separate tissue samples in two lanes).

Mass Spectrometry Identifies p165 as CPS1

Since the p165 protein appeared to be abundant on the basis of immunoreactivity in western blots, we performed immunoprecipitation using Hep Par 1 antibody for the purpose of protein purification. After immunoprecipitation, the protein was eluted and resolved in SDS-PAGE. The gel was then stained with Coomassie blue. As shown in Figure 4, a distinctive p165 kDa band was readily visible. The p165 band was excised from the gel and used for protein identification.

LC–MS/MS analysis was then performed, followed by database search against NCBI_nr using MASCOT for the tryptic digests of the protein from the distinct band within the SDS-PAGE gel. The sequence and mass data of a total 116 peptide fragments matched a protein coded as IPI00011062, which is CPS1, with a high matching score of 2016 (see Table 1). The protein matching score is the sum of the unique ion scores, which is based on the absolute probability (*P*) that the observed match between the experimental data and the database sequence is a random event. The reported score is −10Log (*P*). The nominal molecular mass for the identified protein (Mr) is 165 ,975 kDa and the calculated p*I* is 6.30; the molecular mass is close to the experimental range of the gel band displayed in the SDS-PAGE (Figure 4). The identified peptide sequences covered 36% of the total amino-acid sequence of carbamoyl-phosphate synthase 1. These findings identified the Hep Par 1 antigen (p165) to be carbamoyl-phosphate synthase 1 (CPS1) with a very high degree of probability.

To further confirm that CPS1 is the antigen detected by Hep Par 1 antibody, we performed immunoprecipitation analysis. We precipitated liver protein extract with either Hep Par 1 antibody (Figure 5a) or anti-CPS1 antibody (Figure 5b). The eluted immunoprecipitate products were resolved in SDS-PAGE, followed by transferring to membrane and incubating the membrane with anti-CPS1 antibody (Figure 5a) or Hep Par 1 antibody (Figure 5b). As shown in Figure 5, anti-CPS1 specifically reacted with the Hep Par 1-precipitated 165 kDa protein (Figure 5a) and Hep Par 1 reacted with the 165 kDa protein pulled down by anti-CPS1. Moreover, we stained a hepatocellular carcinoma tissue section with anti-CPS1 antibody, knowing that this specific liver tumor was negative for Hep Par 1 immunoreactivity. As shown in Figure 5c, the tumor cells are negative for CPS1 and the residual normal hepatocytes are strongly positive for CPS1. The granular cytoplasmic staining pattern in the residual hepatocytes is similar to that of Hep Par 1 antibody staining pattern.

As noted in the Introduction, Hep Par 1 antibody also reacts with some non-liver cancer cells, especially the 'hepatoid component'. We therefore examined the CPS1 staining pattern in two types of tumors available to us: a gastric adenocarcinoma with hepatoid differentiation, and several yolk sac tumors of the ovary. As shown in Figure 6a, the hepatoid component of the gastric carcinoma was strongly positive for both Hep Par 1 and CPS1. Similarly, the focal

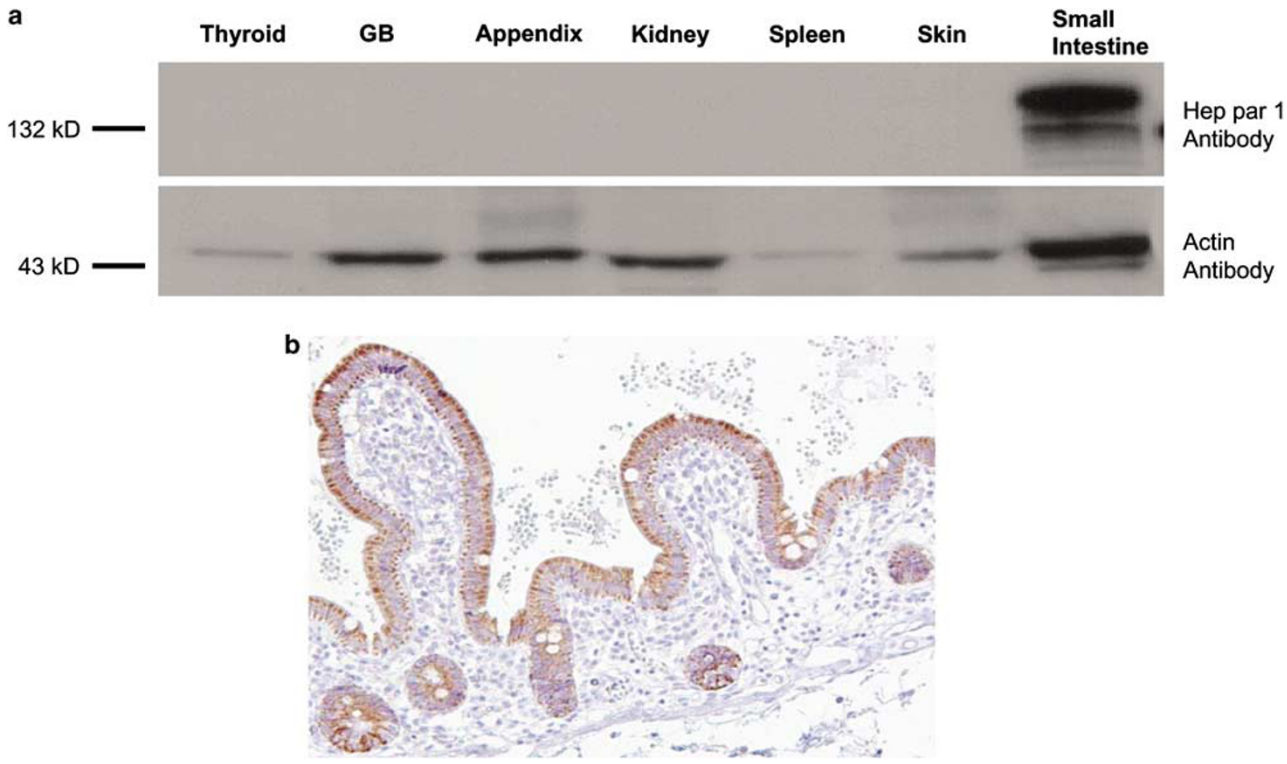


Figure 3 Immunoreactivity of Hep Par 1 antibody of non-liver normal tissues. (**a**) Protein extracts were prepared from normal thyroid, gallbladder (GB), appendix, kidney, spleen, skin, and small intestine, followed by western blot analysis using Hep Par 1 antibody. The Hep Par 1 antibody detects a 165 kDa band in the small intestine tissue only. Actin was used to serve as an internal control. (**b**) Immunohistochemical staining of normal small intestine tissue with Hep Par 1 antibody (1:50 dilution; original magnification $\times$ 200).

Figure 4 Immunoprecipitation of liver extracts and frozen fresh liver tissue. Liver extract was incubated with Hep Par 1 antibody, followed by protein A selection. The protein elute was resolved in 10% SDS-PAGE and stained with Coomassie blue. The 165 kDa protein band was then excised from the gel and used for mass spectrometry analysis. All the four lanes were loaded with same liver tissue extract after immunoprecipitation. The amount difference reflects variations during multiple steps of immunoprecipitation.

Hep Par 1-positive tumor cells in the one yolk sac tumor exhibiting 'hepatoid' features were also positive for CPS1. The two other yolk sac tumors exhibited negative immunoreactivity for both Hep Par 1 and CPS1 (data not shown).

Collectively, these experiments firmly establish the connection between Hep Par 1 and CPS1.

CPS1 Expression is Suppressed at the Transcriptional Level in Human Hepatocellular Carcinoma Cell Lines

Figure 2 shows that Huh-7 cells are negative for Hep Par 1. We then examined the CPS1 expression in other hepatocellular carcinoma cell lines, HepG2 and LH86. As shown in Figure 7a, none of the three hepatocellular carcinoma cell lines express CPS1, while cultured human primary hepatocytes strongly express the CPS1 protein. Total RNA was then extracted from both primary hepatocytes and Huh-7 cells, followed by RT-PCR analysis. As shown in Figure 7b, CPS1 RNA is readily detected in hepatocytes, but not Huh-7 cells. To rule out the possibility of genomic deletion of CPS1 gene in Huh-7 cells, genomic DNA was obtained from both hepatocytes and Huh-7 cells. As shown in Figure 7c, the CPS1 gene is present in both cells. These data indicate that suppression of CPS1 expression occurs at the RNA transcriptional level. The fact that both CPS1 mRNA and protein are expressed in primary cultured human hepatocytes excludes culture conditions *per se* as the reason for down-regulation of CPS1 expression.

DISCUSSION

Hep Par 1 antibody is widely used in surgical pathology practice; however, the protein it reacts with is unknown

Table 1 Mass spectrometric identification of the Hep Par 1 antigen

No.	Ions (*m/z*) observed	Charge status	Mass calculated	% Error	Location	Sequence of matching peptides
1	1382.58	+1	1381.72	−0.01	43–55	AQTAHIVLEDGTK
2	692.79	+2	1381.72	0.13	43–55	AQTAHIVLEDGTK
3	1384.58	+1	1381.72	0.13	43–55	AQTAHIVLEDGTK
4	923.47	+1	922.48	0.00	120–127	YLESNGIK
5	462.87	+2	922.48	0.14	120–127	YLESNGIK
6	1193.69	+1	1192.67	0.00	128–138	VSGLLVLDYSK
7	1193.72	+1	1192.67	0.00	128–138	VSGLLVLDYSK
8	597.81	+2	1192.67	0.08	128–138	VSGLLVLDYSK
9	1195.66	+1	1192.67	0.17	128–138	VSGLLVLDYSK
10	1147.46	+1	1146.55	−0.01	139–147	DYNHWLATK
11	574.69	+2	1146.55	0.07	139–147	DYNHWLATK
12	574.99	+2	1146.55	0.12	139–147	DYNHWLATK
13	1218.69	+1	1216.61	0.09	148–157	SLGQWLQEEK
14	1091.76	+1	1089.58	0.11	158–167	VPAIYGVDTR
15	1102.53	+1	1101.60	−0.01	198–207	QNLIAEVSTK
16	552.27	+2	1101.60	0.09	198–207	QNLIAEVSTK
17	1104.61	+1	1101.60	0.18	198–207	QNLIAEVSTK
18	960.77	+1	959.51	0.03	220–228	VVAVDCGIK
19	962.50	+1	959.51	0.21	220–228	VVAVDCGIK
20	636.43	+3	1904.96	0.07	238–253	RGAEVHLVPWNHDFTK
21	954.19	+2	1904.96	0.07	238–253	RGAEVHLVPWNHDFTK
22	876.03	+2	1748.86	0.07	239–253	GAEVHLVPWNHDFTK
23	1326.59	+1	1324.71	0.07	317–328	GQNQPVLNITNK
24	664.08	+2	1324.71	0.11	317–328	GQNQPVLNITNK
25	1327.68	+1	1324.71	0.15	317–328	GQNQPVLNITNK
26	1015.27	+2	2027.07	0.07	459–477	TVLMNPNIASVQTNEVGLK
27	1023.10	+2	2043.07	0.06	459–477	TVLMNPNIASVQTNEVGLK oxidation (M)
28	737.69	+1	736.38	0.04	548–553	QLFSDK
29	1306.65	+1	1305.68	0.00	576–587	AADTIGYPVMIR
30	797.70	+2	1591.78	0.10	588–603	SAYALGGLGSGICPNR
31	1037.64	+1	1036.51	0.01	604–612	ETLMDLSTK
32	1053.43	+1	1052.51	−0.01	604–612	ETLMDLSTK oxidation (M)
33	528.07	+2	1052.51	0.15	604–612	ETLMDLSTK oxidation (M)
34	1364.65	+1	1363.72	−0.01	613–624	AFAMTNQILVEK
35	1366.65	+1	1363.72	0.14	613–624	AFAMTNQILVEK
36	1380.66	+1	1379.71	0.00	613–624	AFAMTNQILVEK oxidation (M)
37	1037.66	+1	1035.52	0.11	631–638	EIEYEVVR
38	944.68	+1	943.56	0.01	682–689	RTSINVVR
39	473.29	+2	943.56	0.11	682–689	RTSINVVR
40	788.67	+1	787.46	0.03	683–689	TSINVVR
41	1222.61	+1	1221.68	−0.01	729–740	ATGYPLAFIAAK
42	612.52	+2	1221.68	0.11	729–740	ATGYPLAFIAAK
43	875.42	+2	1747.78	0.06	758–772	TSACFEPSLDYMVTK
44	1018.75	+1	1017.53	0.02	794–803	SVGEVMAIGR

Table 1 Continued

No.	Ions (*m/z*) observed	Charge status	Mass calculated	% Error	Location	Sequence of matching peptides
45	1019.67	+1	1017.53	0.11	794–803	SVGEVMAIGR
46	1015.49	+1	1014.47	0.00	804–811	TFEESFQK
47	1015.53	+1	1014.47	0.01	804–811	TFEESFQK
48	715.89	+2	1430.64	−0.06	815–826	MCHPSIEGFTPR
49	1431.51	+1	1430.64	−0.01	815–826	MCHPSIEGFTPR
50	716.79	+2	1430.64	0.07	815–826	MCHPSIEGFTPR
51	1433.48	+1	1430.64	0.13	815–826	MCHPSIEGFTPR
52	724.84	+2	1446.64	0.07	815–826	MCHPSIEGFTPR oxidation (M)
53	565.89	+2	1128.56	0.11	832–840	EWPSNLDLR
54	1492.48	+1	1491.68	−0.01	857–869	AIDDNMSLDEIEK
55	747.36	+2	1491.68	0.07	857–869	AIDDNMSLDEIEK
56	1494.53	+1	1491.68	0.12	857–869	AIDDNMSLDEIEK
57	752.55	+1	751.41	0.02	870–875	LTYIDK
58	756.72	+1	755.40	0.04	876–880	WFLYK
59	862.66	+1	861.43	0.03	883–889	DILNMEK
60	878.53	+1	877.42	0.01	883–889	DILNMEK oxidation (M)
61	1438.61	+1	1437.67	0.00	893–905	GLNSESMTEETLK
62	1454.49	+1	1453.66	−0.01	893–905	GLNSESMTEETLK oxidation (M)
63	795.67	+1	794.38	0.04	909–915	EIGFSDK
64	795.74	+1	794.38	0.05	909–915	EIGFSDK
65	1148.65	+1	1147.57	0.01	920–929	CLGLTEAQTR
66	1150.54	+1	1147.57	0.17	920–929	CLGLTEAQTR
67	893.66	+1	892.49	0.02	936–942	NIHPWVK
68	448.00	+2	892.49	0.17	936–942	NIHPWVK
69	1298.52	+1	1297.66	−0.01	1030–1039	LYFEELSLER
70	1300.65	+1	1297.66	0.15	1030–1039	LYFEELSLER
71	1230.68	+1	1229.64	0.00	1075–1085	IMGTSPLQIDR
72	616.45	+2	1229.64	0.10	1075–1085	IMGTSPLQIDR
73	1232.63	+1	1229.64	0.16	1075–1085	IMGTSPLQIDR
74	624.28	+2	1245.64	0.07	1075–1085	IMGTSPLQIDR oxidation (M)
75	799.70	+1	798.44	0.03	1101–1107	VAQAPWK
76	1419.62	+1	1418.74	−0.01	1108–1120	AVNTLNEALEFAK
77	1419.68	+1	1418.74	0.00	1108–1120	AVNTLNEALEFAK
78	1421.64	+1	1418.74	0.13	1108–1120	AVNTLNEALEFAK
79	1421.68	+1	1418.74	0.14	1108–1120	AVNTLNEALEFAK
80	865.79	+1	864.43	0.04	1151–1157	FLEEATR
81	1236.67	+1	1235.69	0.00	1158–1168	VSQEHPVVLTK
82	619.50	+2	1235.69	0.11	1158–1168	VSQEHPVVLTK
83	1238.66	+1	1235.69	0.16	1158–1168	VSQEHPVVLTK
84	977.44	+1	976.45	0.00	1175–1183	EVEMDAVGK
85	977.52	+1	976.45	0.01	1175–1183	EVEMDAVGK
86	1401.54	+1	1400.71	−0.01	1248–1259	GNDVLVIECNLR
87	1401.60	+1	1400.71	−0.01	1248–1259	GNDVLVIECNLR
88	1403.65	+1	1400.71	0.14	1248–1259	GNDVLVIECNLR

Table 1 Continued

No.	Ions (*m/z*) observed	Charge status	Mass calculated	% Error	Location	Sequence of matching peptides
89	811.71	+1	810.43	0.03	1263–1269	SFPFVSK
90	1278.69	+1	1277.69	0.00	1270–1281	TLGVDFIDVATK
91	640.65	+2	1277.69	0.13	1270–1281	TLGVDFIDVATK
92	1280.67	+1	1277.69	0.15	1270–1281	TLGVDFIDVATK
93	1133.48	+1	1132.54	−0.01	1282–1291	VMIGENVDEK
94	567.81	+2	1132.54	0.10	1282–1291	VMIGENVDEK
95	1135.53	+1	1132.54	0.18	1282–1291	VMIGENVDEK
96	1149.49	+1	1148.54	0.00	1282–1291	VMIGENVDEK oxidation (M)
97	1151.46	+1	1148.54	0.17	1282–1291	VMIGENVDEK oxidation (M)
98	672.03	+3	2012.11	0.05	1292–1309	HLPTLDHPIIPADYVAIK
99	1007.6	+2	2012.11	0.05	1292–1309	HLPTLDHPIIPADYVAIK
100	991.65	+1	990.47	0.02	1310–1317	APMFSWPR
101	496.77	+2	990.47	0.11	1310–1317	APMFSWPR
102	504.97	+2	1006.47	0.15	1310–1317	APMFSWPR oxidation (M)
103	1068.56	+1	1067.61	0.00	1318–1326	LRDADPILR
104	535.39	+2	1067.61	0.11	1318–1326	LRDADPILR
105	799.73	+1	798.42	0.04	1320–1326	DADPILR
106	799.76	+1	798.42	0.04	1320–1326	DADPILR
107	1216.42	+2	2430.07	0.03	1327–1348	CEMASTGEVACFGEGIHTAFLK oxidation (M)
108	854.66	+1	853.44	0.03	1349–1356	AMLSTGFK
109	870.72	+1	869.43	0.03	1349–1356	AMLSTGFK oxidation (M)
110	743.46	+2	1483.86	0.07	1361–1373	GILIGIQQSFRPR
111	795.69	+2	1587.80	0.10	1374–1387	FLGVAEQLHNEGFK
112	1162.58	+1	1161.59	0.00	1445–1453	FVHDNYVIR
113	1163.63	+1	1161.59	0.09	1445–1453	FVHDNYVIR
114	905.60	+1	904.50	0.01	1472–1479	LFAEAVQK
115	905.60	+1	904.50	0.01	1472–1479	LFAEAVQK
116	907.41	+1	904.50	0.21	1472–1479	LFAEAVQK

Note: The protein score is calculated by combination of the experimental data (the table) and database searching.

despite a decade-and-a-half of clinical use. Our goal was to identify this protein. After confirming the presence of antigen that reacts with Hep Par 1 antibody in fresh liver tissue, we performed immunoprecipitation, which allowed us to identify a 165 kDa protein in the liver tissue. After purification of the p165 protein, we analyzed the protein using a standard mass spectrometry method. As shown in Table 1, the majority of digested peptides correspond to CPS1.

To more definitively establish this link, we performed immunoprecipitation–western blot analysis, whereby protein immunoprecipitated with Hep Par 1 was immunoreactive on western blot with anti-CPS1, and protein immunoprecipitated with anti-CPS1 was immunoreactive on western blot with Hep Par 1 (Figure 5). Immunohistochemical staining of non-liver tumors immunoreactive for Hep Par 1 (one gastric carcinoma and one yolk sac tumor) also demonstrated immunoreactivity for CPS1 in the same region of the tumors (Figure 6). We cannot totally exclude the possibility that Hep Par 1 antibody may also bind to other proteins. However, the evidence in this report supports the notion that Hep Par 1 predominantly binds to CPS1.

CPS1 is a rate-limiting enzyme in urea cycle.[7,21,22] Converting ammonia to urea is one of the essential functions of the liver. CPS1 is an abundant hepatocellular protein and predominantly localizes in the mitochondrion, consistent with the granular immunohistochemical staining pattern of the Hep Par 1 antibody. Transcription of the CPS1 gene is subject to physiological regulation[23–25] and a hepatotoxic

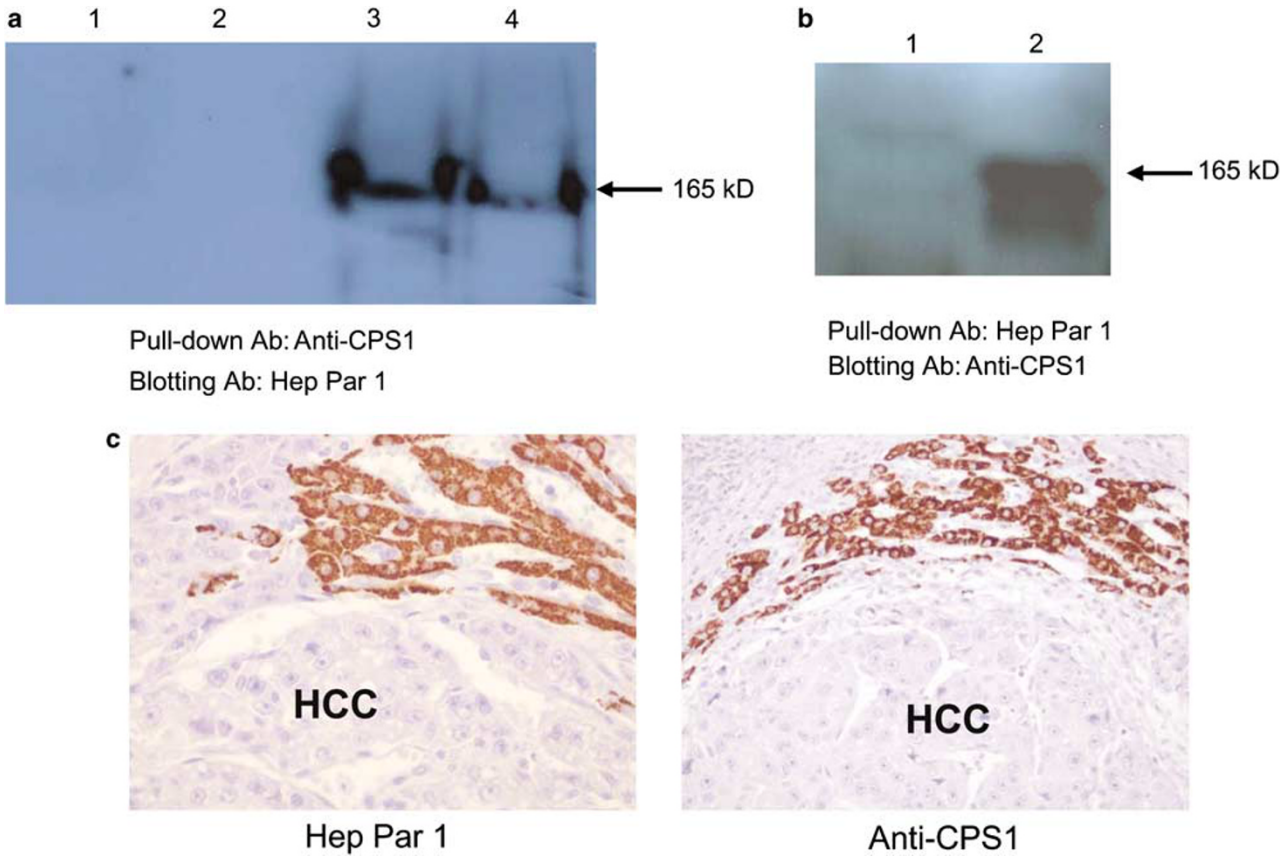


Figure 5 Hep Par 1 antibody and CPS1 recognize p165 protein. (**a**) Hep Par 1 immunoprecipitated protein reacts with anti-CPS1 antibody. Liver extract was incubated with control immunoglobulin (lanes 1 and 2) or Hep Par 1 antibody (lanes 3 and 4) and protein A selection. The protein elute was then analyzed by western blot using an anti-CPS1 polyclonal antibody. (**b**) Liver extract was first incubated with control immunoglobulin (lane 1) and anti-CPS1 antibody (Lane 2), followed by protein A selection and western blot analysis with Hep Par 1 antibody (lane 2). (**c**) Immunohistochemical staining for CPS1 of hepatocellular carcinoma tissue with negative Hep Par 1 staining. The tumor cells are negative for anti-CPS1; residual normal hepatocytes in the surrounding cirrhotic liver are strongly positive for Hep Par 1 and anti-CPS1. Magnification, $\times 400$.

dose of acetaminophen can inhibit CPS1 activity.[26] CPS1 deficiency is a genetic syndrome that severely affects development due to increased level of ammonia.[27] In some of these patients, the liver may show focal glycogenosis.[28]

It has been reported that CPS1 can be released from the hepatocytes during liver injury.[29,30] However, the clinical application of the elevated serum CPS1 remains to be determined. More importantly, the connections between altered CPS1 expression in human hepatocytes in disease conditions, especially hepatocarcinogenesis, has not been explored. It is possible that CPS1 alteration may be a surrogate marker to reflect the function of normal hepatocytes or cancerous 'well-differentiated' hepatocytes. The identification of CPS1 as the antigen for Hep Par 1 antibody now justifies investigation of the potential role of CPS1 in the pathobiology of the liver, especially hepatocarcinogenesis. In particular, it will be valuable to determine whether CPS1 expression plays a causal role in the differentiation state of tumorous hepatocytes and non-liver cells, or is instead only a surrogate marker for hepatocellular differentiation. Even in the latter instance, determining what regulates CPS1 gene expression may be a valuable future line of investigation.

There are two additional interesting observations in our study: (1) the presence of CPS1 in small intestinal epithelium; (2) the absence of CPS1 in a cultured hepatocellular carcinoma cell lines with the suppression occurring at the transcriptional level. Since CPS1 is an enzyme in the urea cycle, the intriguing question is whether small intestinal epithelial cells contain a functional urea cycle, or whether the presence of CPS1 denotes a previously unappreciated nitrogen-processing pathway in small intestinal epithelium, as suggested by studies using pig enterocytes.[31,32] Future experiments should be considered to examine the other urea cycle enzymes in the small intestinal epithelium. Importantly, examination of ammonia metabolism within the small intestine epithelium could provide important insights into intestinal epithelial physiology, particularly given a recent report that CPS1 polymorphisms constitute a risk condition for neonatal necrotizing enterocolitis.[33]

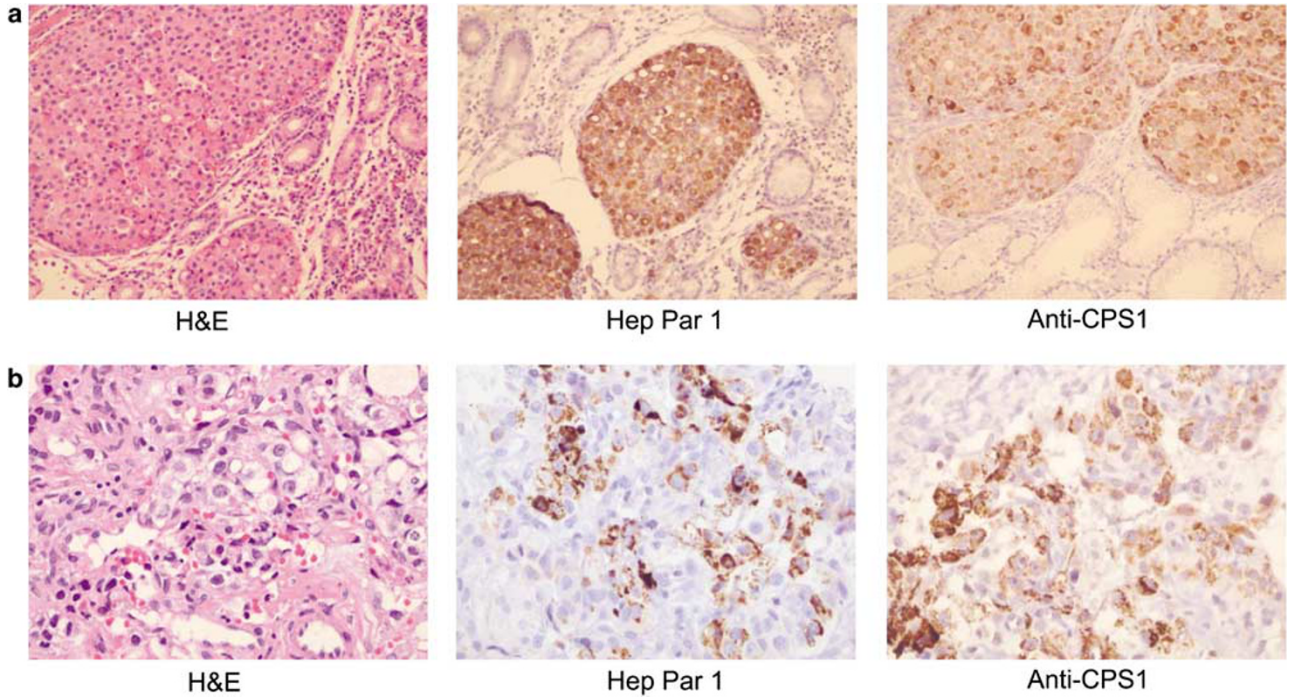
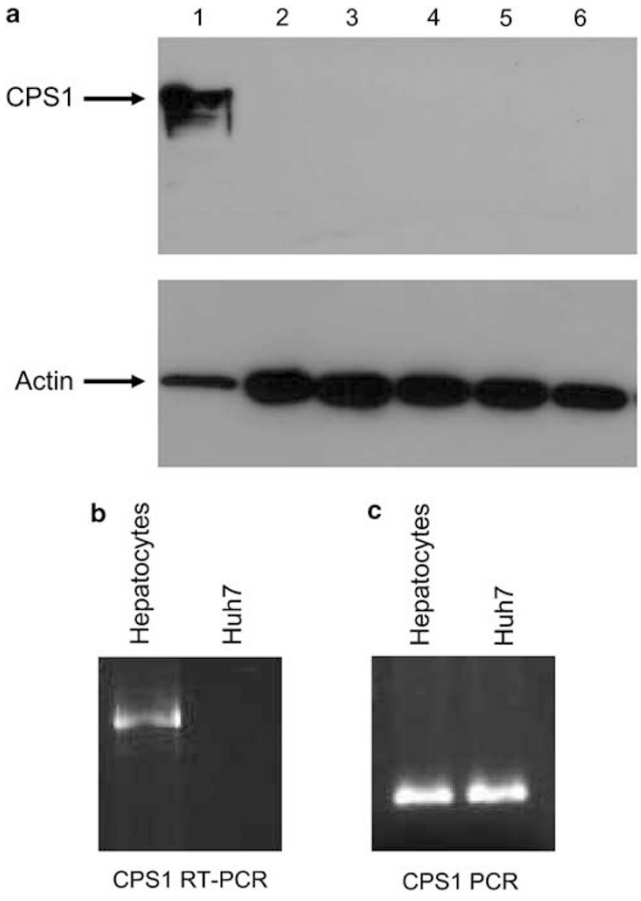


Figure 6 Hep Par 1 and CPS1 have similar immunostaining pattern in non-liver tumors. (**a**) A 'hepatoid' gastric carcinoma shows immunoreactivity with both Hep Par 1 and anti-CPS1 antibodies (magnification, $\times 200$). (**b**) A yolk sac tumor showing same group of cells with both CPS1 and Hep Par 1 immunoreactivity (magnification, $\times 400$).



The absence of CPS1 mRNA and protein in the three hepatocellular carcinoma cell lines suggests that CPS1 is only expressed in more differentiated HCC, recognizing that expression of highly differentiated cellular proteins may be downregulated in immortalized cell lines. In fact, clinical studies have demonstrated that Hep Par 1 does not appear to react with poorly differentiated HCC.[34,35] A recent study has shown that CPS1 gene transcription is downregulated in liver cancer tissues.[36] However, the absence of CPS1 in the cancer cell lines cannot be explained entirely by the hepatocellular differentiation state. It is known that all three cell lines express abundant levels of albumin and α-1 antitrypsin, indicating that many liver-specific transcription factors are present in these cell lines. The total absence of CPS1 in these cell lines is highly suggestive of other regulatory mechanisms of CPS1 gene expression control, such as epigenetic regulation. Future experimentation is needed to reveal the mechanisms.

Figure 7 Cultured hepatocellular carcinoma cell lines do not have CPS1 expression. (**a**) Western blot analysis of cellular extracts from cultured cells using Hep Par 1 antibody. Lane 1: primary hepatocytes in culture; lanes 2–4: three different clones of Huh-7 cells; lane 5, HepG2 cells; lane 6: LH86 cells. The figure shows that CPS1 is expressed only in primary cultured hepatocytes. Actin serves as an internal control. (**b**) CPS1 mRNA expression in normal hepatocytes and Huh-7 cells using RT-PCR analysis; only primary cultured hepatocytes express CPS1 mRNA. (**c**) CPS1 gene in primary cultured hepatocytes and Huh-7 cells using PCR analysis of genomic DNA. Gene status is intact in both primary cultured hepatocytes and Huh-7 cells.

The pathophysiological or functional significance of CPS1 in cancer cell differentiation and development remains to be determined. Given that disposal of excess nitrogen via the urea cycle is essential for life, underexpression of this key enzyme of the urea cycle raises intriguing questions about the nitrogen balance of malignant tumors. For the most part, only HCC express Hep Par 1 (and, hence, CPS1). We do not know whether function of the urea cycle has any role to play in carcinogenesis, or whether such function may prove to be of benefit for treatment of these recalcitrant tumors.

In conclusion, our study has identified CPS1 as the antigen reacting with the Hep Par 1 antibody. CPS1 is expressed in human hepatocytes and small intestinal epithelium, with silenced expression in human hepatocellular carcinoma cell lines at the gene transcriptional level. Our findings should promote further investigations of CPS1 in the context of underlying liver diseases and hepatocarcinogenesis.

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