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Positron Emission Tomography of Human Hepatocellular Carcinoma Xenografts in Mice Using Copper (II)-64 Chloride as a Tracer

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Abstract

Rationale and Objectives—The aim of this study was to assess copper metabolism of human hepatocellular carcinoma (HCC) with positron emission tomographic (PET) imaging using copper (II)-64 chloride ($^{64}\text{CuCl}_2$) as a tracer.

Materials and Methods—PET imaging of athymic mice ($n = 5$) bearing extrahepatic HCC xenografts was performed 24 hours after the intravenous injection of $^{64}\text{CuCl}_2$, followed by ex vivo tissue radioactivity assay. Expression of human copper transporter 1 (hCTR1) in HCC cells and tissues was examined by real-time reverse transcription polymerase chain reaction and immunohistochemistry analysis, respectively.

Results—The extrahepatic HCC xenografts in mice with increased uptake of ^{64}Cu radionuclide were visualized on the micro-PET images obtained 24 hours after the intravenous injection of $^{64}\text{CuCl}_2$. PET quantitative analysis revealed increased ^{64}Cu radioactivity in tumor tissues (2.7 ± 0.6 %ID/g) compared to that in the soft tissue of the left shoulder opposite to the tumor site (0.6 ± 0.2 %ID/g) and the brain (0.7 ± 0.1 %ID/g) but lower than that of the liver (16.6 ± 1.3 %ID/g). Expression of hCTR1 in the HCC cells and xenograft tumor tissues was demonstrated by real-time reverse transcription polymerase chain reaction and immunohistochemistry analysis, respectively. The expression level of hCTR1 in the Hep3B HCC xenograft tissues was lower than that detected in the normal hepatic tissues and the tissue samples of well-differentiated primary HCC. Variable expression of hCTR1 was detected in the tissue samples of moderately differentiated primary HCC.

Conclusions—Extrahepatic human HCC xenografts in mice could be localized with $^{64}\text{CuCl}_2$ PET imaging, which might be useful for the localization and quantitative assessment of copper metabolism in extrahepatic metastases of HCC in humans.

Keywords

Positron emission tomography; copper(II)-64 chloride; hepatocellular carcinoma; copper metabolism; human copper transporter 1

Hepatocellular carcinoma (HCC) is a threat to the health of the global population, particularly to people living in Asia and sub-Saharan Africa (1,2). Significant advances have been made in the treatment of patients diagnosed with the early stages of HCC, but the prognosis for patients with extrahepatic HCC metastases is poor, because they are often resistant to systemic chemotherapy (3,4). Moreover, the prognosis of patients with intracranial metastases of HCC is dismal (5,6). Liver transplantation is a potentially curative treatment for those patients with HCC localized within the liver (7). In the pre-transplantation workup, however, it is essential to exclude extrahepatic metastases in those patients who are considered candidates for liver transplantation. Positron emission tomographic (PET) imaging using 2-deoxy-2-[^{18}F]-fluoro-D-glucose (^{18}F -FDG) is clinically well accepted for the staging of many human cancers and has been found to be highly sensitive for the detection of the extrahepatic spread of hypermetabolic HCC (8,9). However, ^{18}F -FDG PET imaging is limited for the detection of intracranial HCC metastases because of the high background of FDG uptake by the normal brain tissue. Thus, it is necessary to establish an alternative tracer that may be used for the evaluation of intracranial metastases of HCC under circumstances in which the use of ^{18}F FDG PET imaging is limited.

Copper is an essential nutrient in mammals, and intracellular copper homeostasis in humans is regulated by a delicate network of copper transporters (10), which include human copper transporter 1 (hCTR1), ATP7A, ATP7B, as well as copper chaperons antioxidant protein 1, cytochrome c oxidase 17, and copper chaperone for superoxide dismutase (11–16). Copper is required for cell proliferation and tumor growth, and high concentrations of copper have been observed in many of human tumor tissues (17–19). Extrahepatic mouse hepatoma grafts and human prostate xenografts in mice could be localized with PET imaging using $^{64}\text{CuCl}_2$ as a tracer (20,21). In this study, PET imaging of athymic mice bearing human HCC xenografts was conducted to determine whether extrahepatic human HCC metastases could be localized with $^{64}\text{CuCl}_2$ PET imaging. To study the molecular mechanism of copper hypermetabolism in HCC, expression of hCTR1 in HCC cells and tumor tissues was examined by quantitative real-time reverse transcription polymerase chain reaction (RC-PCR) and immunohistochemistry analysis, respectively.

MATERIALS AND METHODS

Cells and Animal Model

Hep3B human HCC cells and HEK293 human embryonic kidney cells (ATCC, Manassas, VA) were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. PZ-HPV-7 immortalized prostate epithelial cells (a gift from Dr Jer-Tsong Hsieh, University of Texas Southwestern Medical Center) were cultured in prostate epithelial basal medium supplemented with PrEGM SingleQuot growth factor (Lonza, Walkersville, MD), 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. PC-3 human prostate cancer cells (ATCC) were cultured in T medium supplemented with 10% fetal bovine serum, 100 U/mL, and 100 mg/mL streptomycin. Athymic nu/nu mice (female, 5 to 6 weeks old) were purchased from Harlan Laboratory (Indianapolis, IN) and housed in the facility according to the guidelines and a protocol approved by the Animal Investigation Committee at Wayne State University. To establish extrahepatic hepatoma xenografts, 5×10^6 Hep3B cells were injected to the right shoulder of the mice subcutaneously approximately 3 weeks prior to the micro-PET imaging.

PET Imaging

PET imaging of five mice bearing HCC xenografts was performed using a micro-PET R4 tomograph (Concorde Microsystems, Knoxville, TN) in a protocol described previously (20,21). Briefly, the mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (7 mg/kg) and imaged at 24 hours after the intravenous injection of $^{64}\text{CuCl}_2$ ($2 \mu\text{Ci/g}$ body weight) as a bolus injection through the tail vein. The radiopharmaceutical $^{64}\text{CuCl}_2$ was provided by the Mallinckrodt Institute of Radiology, Washington University School of Medicine (St Louis, MO). A whole-body data acquisition was acquired, consisting of two overlapping frames of 15 minutes in duration (2-cm overlap). The whole-body images were corrected for attenuation using the previously acquired transmission scans and reconstructed using an iterative ordered subset expectation maximization two-dimensional algorithm (22).

PET Quantitative Analyses of Tracer Concentration

PET quantitative analysis was performed to assess tissue tracer concentration as described previously (23). Briefly, the PET data were reconstructed using measured attenuation, scatter correction, and the ordered subset expectation maximization two-dimensional iterative algorithm, yielding an isotropic spatial resolution of about 2 mm full width at half maximum. The images were subsequently processed using ASIPRO PET data analysis software (Concorde Microsystems). Regions of interest (ROIs) were drawn in all planes over the tumor, liver, brain, and soft tissue region on the left flank opposite the tumor. The average tracer concentration (kilobecquerels [millicuries] per cubic centimeter) for each ROI was obtained as a weighted (by area) average of the tracer concentration obtained from all image planes in which ROIs for that particular tissue region were defined. A calibration factor predetermined by scanning a phantom was used to convert counts per pixel per minute to kilobecquerels per cubic centimeter for the tracer $^{64}\text{CuCl}_2$. Finally, the decay-corrected percentage of injected dose (ID) per gram of tissue for each ROI was calculated by dividing the obtained average tracer concentration (kilobecquerels per cubic centimeter) in the region by the total ID (kilobecquerels).

Radioactivity Assay of Tissue Radiotracer Concentration

Upon completion of $^{64}\text{CuCl}_2$ PET imaging, the tumor-bearing mice were euthanized under anesthesia, and postmortem mouse and tumor tissues were harvested, weighted, and counted for radioactivity with a Packard Cobra II Gamma counter (Perkin-Elmer, Wellesley, MA). Tissue tracer concentrations were calculated and recorded as a decay-corrected percentage of ID per gram of tissue.

Quantitative Real-time RT-PCR

Quantitative real-time RT-PCR was conducted to assess transcriptional activity of hCTR1 in Hep3B cells in a method described previously by Xie et al (24). Briefly, total cellular ribonucleic acid was isolated using a Trizol kit from Qiagen (Valencia, CA), followed by reverse transcription and complementary deoxyribonucleic acid synthesis with a Superscript cDNA Synthesis kit (Invitrogen, Carlsbad, CA). Real-time RT-PCR was performed with primers specific for human hCTR1 (5'-CACCATGGATCATTCCCACCATATGGG-3' and 5'-TCCAGCTGTATTGATCACCAA ACC-3'), using Q SYBR Green Supermix kit (Bio-Rad, Hercules, CA). Human 18s ribonucleic acid primers (5'-GGAATTGACGGAAGGGCACCACC-3' and 5'-GTGCAGCCCCGGACATCTAAGG-3') were used as quantitative control of real-time RT-PCR. RT-PCR products were separated on a 1% trisborate ethylenediaminetetraacetic acid/agarose gel, and quantitative values of messenger ribonucleic acid (mRNA) level against control (relative values) were calculated with MyiQ system software (Bio-Rad).

IHC Analysis

IHC analysis of hCTR1 expression was conducted using the Hep3B xenograft tissues, the archived tissues samples of human primary HCC from the tissue bank, and control normal or nonmalignant hepatic tissues, in a protocol approved by the institutional review board. To examine the expression of hCTR1, 5- μ m tissue sections were prepared with postmortem xenograft tumor tissues fixed in 10% buffered formalin, microwave-treated in citrate buffer at pH 5 for 5 minutes for antigen retrieval, and incubated with a polyclonal antibody specific for hCTR1 at a dilution of 1:250 (Novus Biologicals, Littleton, CO). Following incubation with primary antibody, the immunoreactivity against hCTR1 was visualized by reaction with horseradish peroxidase-labeled goat-antirabbit or antimouse secondary antibodies (Vector Laboratories, Burlingame, CA). Tissue sections incubated with normal rabbit serum were used as a negative control. Immunoreactivity of hCTR1 was visually examined and recorded either as negative or 1+ (mild), 2+ (moderate), or 3+ (intense). Microscopic images of the stained sections were recorded with an Olympus microscope equipped with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI).

Statistical Analysis

Statistical analysis of PET data was performed as described previously (16). Data of quantitative PET analysis and ex vivo radioactivity count are expressed as mean $\pm$ standard deviation. To determine whether tracer uptake observed using PET imaging (percentage of ID per gram of tissue) differed significantly from tracer uptake of ^{64}Cu determined using radioactivity assay ex vivo, a 2×4 repeated-measures analysis of variance was performed, in which the between-subjects factor was the test (PET imaging or ex vivo assay) and the within-subjects factor represented the four ROIs (tumor, liver, brain, and soft tissue). A significant overall *F* value was followed by a limited number of post hoc tests comparing tracer uptake in tumor with tracer uptake in each of the other tissues. Because the number of post hoc comparisons (3) for each outcome was less than the number of levels of the repeated factor (5), no correction for multiple comparisons was used. *P* values $<.05$ were considered to represent statistical significance. The quantity of RT-PCR products was expressed as the mean and standard deviation of relative values against control. To determine whether hCTR1 mRNA levels in Hep3B cells were different from those in other cells, Student's *t* tests were performed. Again, *P* values $<.05$ were considered to represent statistical significance.

RESULTS

Visualization of Hep3B HCC Xenografts in Mice with $^{64}\text{CuCl}_2$ PET Imaging

Human HCC xenografts in mice were well visualized on the micro-PET images obtained 24 hours after the intravenous injection of the tracer $^{64}\text{CuCl}_2$ (Fig 1). Intense ^{64}Cu radioactivity was observed in the liver, along with diffuse excretory ^{64}Cu radioactivity present in the abdomen.

Biodistribution of ^{64}Cu Radioactivity by PET Quantitative Analysis

PET quantitative analysis revealed increased uptake of ^{64}Cu by the tumor (2.7 ± 0.6 %ID/g) compared with much lower ^{64}Cu radioactivity in the shoulder muscles (0.6 ± 0.2 %ID/g) and the brain (0.7 ± 0.1 %ID/g). There was intense ^{64}Cu radioactivity in the liver (16.6 ± 1.3 %ID/g), along with diffuse ^{64}Cu radioactivity in the abdomen derived from hepatobiliary clearance of the tracer from the liver. Following a significant region effect ($P < .001$), post hoc tests of PET quantitative analysis data revealed that ^{64}Cu radioactivity of the tumor (2.7 ± 0.6 %ID/g) was significantly higher than that in shoulder muscle (0.6 ± 0.2 %ID/g) ($P = .$

004) as well as the brain (0.7 ± 0.1 %ID/g) ($P = .004$) but significantly lower than that in the liver (16.6 ± 1.3 %ID/g) ($P = .002$).

Biodistribution of ^{64}Cu Radioactivity by Ex Vivo Tissue Radioactivity Assay

The results of tissue radioactivity assays demonstrated increased ^{64}Cu radioactivity of tumor tissues (2.8 ± 0.6 %ID/g), as shown in Figure 2. As expected, the mouse liver contained the highest concentration of the tracer (18.8 ± 3.0 %ID/g), followed by the kidneys (6.8 ± 1.3 %ID/g), lungs (6.4 ± 1.7 %ID/g), intestines (6.4 ± 0.7 %ID/g), heart (3.4 ± 1.0 %ID/g), and spleen (2.4 ± 0.6 %ID/g). In contrast, very low tracer concentrations were determined in both the shoulder muscle (0.9 ± 0.5 %ID/g) and brain (0.5 ± 0.2 %ID/g). Radioactivity of lung tissues was artificially high because of collapse of the lungs at the time of ex vivo tissue radioactivity assay by gamma count. Following a significant region effect ($P < .001$), post hoc tests showed that tracer concentration in tumor tissue was significantly higher than that in both the shoulder muscle ($P = .003$) and brain ($P = .001$) but lower than the concentrations in the liver ($P = .001$) and the kidneys ($P = .018$). Finally, no significant differences were determined between tumor tissue, spleen ($P = .22$), and heart tissue ($P = .86$). Repeated-measures analysis of variance showed a nonsignificant region-by-test interaction ($P = .23$), indicating that the two tests (in vivo PET imaging and ex vivo measurement of tracer concentration) were similar.

High-level Transcription of hCTR1 in Hep3B Cells by Real-time RT-PCR

Quantitative real-time RT-PCR was performed to determine hCTR1 transcriptional activity in the Hep3B HCC cells, compared to that in nonmalignant PZ-HPV-7 and HEK293 cells, as well as PC-3 prostate cancer cells (Fig 3). Quantitative data from real-time RT-PCR showed that the mean hCTR1 mRNA level in the Hep3B cells was 8.14 ± 0.28 times higher than that in the PZ-HPV-7 cells ($P = .008$). As expected, a high level of hCTR1 mRNA was also detected in the PC-3 cells, which was 9.0 ± 1.2 times higher than that in the PZ-HPV-7 cells ($P = .008$). Additionally, a high level of hCTR1 mRNA was also detected in the HEK293 cells, immortalized human embryonic kidney cells transformed by sheared adenovirus type 5 deoxyribonucleic acid, which was 6.32 ± 0.78 times higher than that detected in the PZ-HPV-7 cells ($P = .007$).

Detection of hCTR1 Expression in Hep3B and Primary HCC Tissues by IHC Analysis

hCTR1 immunoreactivity was detected on the Hep3B xenograft tumor tissues following incubation of the tissue sections with the hCTR1-specific antibody (Fig 4a), which is nonhomogeneous and less intense compared to the hCTR1 immunoreactivity detected in control normal hepatic tissues (Fig 4b). Furthermore, IHC analysis of hCTR1 demonstrated variable hCTR1 immunoreactivity in the tissue samples from the patients diagnosed with HCC or other benign liver disease, compared to hCTR1 immunoreactivity in the normal hepatic tissues (Table 1, Fig 5). Strong hCTR1 immunoreactivity was detected in the tissue samples from four patients diagnosed with moderately differentiated HCC (Table 1, Fig 5a) and two patients diagnosed with well-differentiated HCC (Table 1, Fig 5c) at an intensity similar to that observed in the normal hepatic tissues (Fig 5d). In contrast, hCTR1 immunoreactivity in the tissue samples from other three patients diagnosed with moderately differentiated HCC (Table 1, Fig 5b) was lower than that detected in the normal or nonmalignant hepatic tissues (Table 1). In addition, relatively lower hCTR1 immunoreactivity was also observed in the hepatic tissue from a patient diagnosed with liver cirrhosis associated with steatosis (Table 1).

DISCUSSION

Extrahepatic human HCC xenografts with increased ^{64}Cu radioactivity were well visualized on micro-PET images obtained 24 hours after the intravenous administration to the tumor-bearing mice of $^{64}\text{CuCl}_2$ via the tail vein. Overall, the results of PET quantitative analysis of tissue ^{64}Cu radioactivity in vivo were similar to those determined by ex vivo tissue radioactivity assay. Small differences between the ^{64}Cu radioactivity of the HCC xenograft tissues measured by PET quantitative analysis ($2.7 \pm 0.6\%$ ID/g) and the tissue ^{64}Cu radioactivity by ex vivo tissue radioactivity assay ($2.8 \pm 0.6\%$ ID/g) were most likely derived from expected technical variation. In contrast to high background cerebral radioactivity in ^{18}F -FDG PET imaging, there was very low background ^{64}Cu radioactivity in the brain tissues of the tumor-bearing mice injected with $^{64}\text{CuCl}_2$ intravenously. These findings suggest that $^{64}\text{CuCl}_2$ PET imaging is expected to be useful for localization of intracranial HCC metastasis for which the use of ^{18}F -FDG PET imaging is limited by high background ^{18}F -FDG activity in the cerebral cortex. However, the use of $^{64}\text{CuCl}_2$ PET imaging for localization of extrahepatic HCC metastases in the abdomen might be limited by the presence of background ^{64}Cu radioactivity in the bowels due to hepatobiliary clearance of the tracer $^{64}\text{CuCl}_2$ injected intravenously. On the other hand, the biodistribution of ^{64}Cu in mice injected with $^{64}\text{CuCl}_2$ may slightly differ from that in humans injected with this tracer. For example, cerebral ^{64}Cu radioactivity in humans may be higher than that in mice after the intravenous injection of $^{64}\text{CuCl}_2$. This needs to be tested in a clinical trial to validate the feasibility and utility of $^{64}\text{CuCl}_2$ PET imaging for the localization of extrahepatic HCC metastases in humans.

Positron-emitting $^{64}\text{CuCl}_2$ was previously used for nuclear imaging of copper metabolism in patients with Wilson's disease or other liver disease (25–28). The doses of $^{64}\text{CuCl}_2$ used in those studies ranged from 5 to 10 mCi, which did not cause significant normal organ toxicity, such as hepatic toxicity. Because ^{64}Cu radionuclide emits β^+ and β^- particles (29), it is essential to establish a safe dose of $^{64}\text{CuCl}_2$ for PET imaging of HCC in humans. A recently conducted preclinical radiation dosimetry study of $^{64}\text{CuCl}_2$ in an ATP7B^{-/-} knockout mouse model of Wilson's disease predicted a safe dose of $^{64}\text{CuCl}_2$ of 5 MBq/kg (0.14 mCi/kg) for PET imaging of copper metabolism in humans (30). The data from these preclinical studies provide strong support for a clinical trial of $^{64}\text{CuCl}_2$ PET imaging for localizing intracranial metastases in patients diagnosed with advanced HCC.

Copper is required for cell proliferation and tumor angiogenesis (17). However, the molecular mechanism of copper hypermetabolism in tumor growth and angiogenesis is poorly understood. HCTR1 is a high-affinity copper influx transporter, which mediates the cellular uptake of copper in humans (11). To study the molecular mechanism of copper hypermetabolism in HCC, expression of hCTR1 in HCC cells was examined at the transcriptional level with quantitative real-time RT-PCR, because no antibody suitable for immunoblot analysis of hCTR1 is currently available. High hCTR1 mRNA levels were detected in the Hep3B cells and PC-3 prostate cancer cells compared to that in the nonmalignant PZ-HPV-7 prostate epithelial cells, suggesting that hCTR1 mediates increased uptake of ^{64}Cu radionuclide by the Hep3B tumor xenografts in mice visualized on the images in the present study, as well as that by the PC-3 tumor tissues seen on prior micro-PET images (21). High levels of hCTR1 mRNA were also detected in the HEK293 cells, a human embryonic kidney cell line transformed by sheared adenovirus type 5 deoxyribonucleic acid, which is not unexpected considering the kidney origin of this cell line and the fact that hCTR1 is highly expressed in human kidney tissues (31). Strong hCTR1 immunoreactivity in the Hep3B xenograft tissues (Fig 4a) indicated retention of hCTR1 expression in the extrahepatic HCC tumors (Fig 4b). Variable hCTR1 immunoreactivity was detected in the tissues of primary HCC, with strong hCTR1

immunoreactivity in the tissues of two well-differentiated HCCs and four of seven moderately differentiated HCCs (Table 1). In an IHC study of hCTR1 by Holzer et al (31), all of 13 normal hepatic tissue samples were negative for hCTR1 immunostaining. In contrast, mild to intense hCTR1 immunoreactivity was observed on the six normal or benign hepatic tissue samples in the present study (Table 1). This may be related to differences of tissue samples or material and methods of IHC analysis. Additional studies with large numbers of tissue samples are needed to determine a profile of hCTR1 expression in HCC and normal hepatic tissues, to elucidate the role of hCTR1 in pathogenesis of HCC.

On the basis of the requirement of copper for cell proliferation and tumor angiogenesis, trientine, a copper chelator used to treat Wilson's disease, was tested for anticancer treatment of HCC and showed significant antitumor growth and antiangiogenic effects on HCC in animal models (32,33). Considering variable expression of hCTR1 in HCC tissues, $^{64}\text{CuCl}_2$ PET imaging may be used to assess copper metabolism of HCC and select patients with HCC hypermetabolic in copper metabolism for individualized copper-lowering therapy with trientine or other copper chelators, such as tetrathiomolybdate and D-penicillamine.

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Figure 1. Micro-positron emission tomographic (PET) imaging of human hepatocellular carcinoma (HCC) xenografts in mice with $^{64}\text{CuCl}_2$. The HCC xenograft with increased ^{64}Cu radioactivity was well visualized (*arrow*) on a representative micro-PET image of a tumor-bearing mouse injected with $^{64}\text{CuCl}_2$ intravenously. Color scale indicates tracer concentrations in percentage of injected dose per gram of tissue (%ID/g).

Figure 2. Distribution of tissue ^{64}Cu radioactivity as determined by ex vivo tissue radioactivity assay in athymic mice bearing human hepato-cellular carcinoma xenografts. Postmortem tissues were harvested from the tumor-bearing mice ($n = 5$) 24 hours after the intravenous injection of $^{64}\text{CuCl}_2$. The tracer concentration of the tumor was significantly higher than that of the muscular tissue and the brain but much less than that measured from the liver. The error bars represent the standard deviation. %ID/g, percentage of injected dose per gram of tissue.

Figure 3.

Transcriptional activity of human copper transporter 1 (hCTR1) in Hep3B hepatocellular carcinoma (HCC) cells by real-time reverse transcription polymerase chain reaction (RT-PCR). Quantitative real-time RT-PCR analysis demonstrated that hCTR1 messenger ribonucleic acid (mRNA) levels in the Hep3B HCC cells and the PC-3 prostate cancer cells were significantly higher than that in the nonmalignant PZ-HPV-7 prostate epithelial cells. Abundant hCTR1 mRNA molecules were also detected in the control HEK293 human embryonic kidney cells. Transcriptional activity of hCTR1 determined by quantitative real-time RT-PCR was graphed as relative values against that in the control PZ-HPV-7 cells. The error bars represent the standard deviation.

Figure 4.

Expression of human copper transporter 1 (hCTR1) in Hep3B xenograft tumor tissues by immunohistochemistry analysis. Nonhomogeneous hCTR1 immunoreactivity (*brown color* shows horseradish peroxidase product) was detected in the tissue sections of postmortem Hep3B hepatocellular carcinoma xenografts (**a**), compared to homogeneous hCTR1 immunoreactivity visualized on the normal hepatic tissues (**b**). The tissue sections were counterstained with 4', 6-diamidino-2-phenylindole to show cell nuclei in blue. Scale bar = 50 μm .

Figure 5.

Expression of human copper transporter 1 (hCTR1) in primary hepatocellular carcinoma (HCC) tissues by immunohistochemistry analysis. Intense hCTR1 immunoreactivity (*brown color*) shows horse radish peroxidase product) was detected in the tissue sample of a moderately differentiated HCC (**a**), compared to that observed in the tissue of a well-differentiated HCC (**c**) and normal hepatic tissue (**d**). In contrast, only mild hCTR1 immunoreactivity was detected in the tissue sample of another moderately differentiated HCC (**b**). The tissue sections were counterstained with 4', 6-diamidino-2-phenylindole to show cell nuclei in blue. Scale bar = 50 μ m.

TABLE 1

Expression of hCTR1 in Malignant and Nonmalignant Hepatic Tissues by IHC Analysis

Subject	Histopathologic Diagnosis	hCTR1 Immunoreactivity by IHC*
1	HCC, well differentiated	+++
2	HCC, well differentiated	+++
3	HCC, moderately differentiated	+++
4	HCC, moderately differentiated	+++
5	HCC, moderately differentiated	+++
6	HCC, moderately differentiated	+
7	HCC, moderately differentiated	+
8	HCC, moderately differentiated	++
9	HCC, moderately differentiated	+
10	Normal liver parenchyma	+++
11	Normal liver parenchyma	+++
12	Mucinous cystadenoma with steatosis	++
13	Benign liver hemangioma with mild portal inflammation	++
14	Cirrhotic liver with steatosis and portal inflammation	+
15	Benign liver parenchyma	++

HCC, hepatocellular carcinoma; hCTR1, human copper transporter 1; IHC, immunohistochemistry.

* Intensity of hCTR1 immunoreactivity: + = mild, ++ = moderate, +++ = intense.