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Cystine/glutamic acid transporter is a novel marker for predicting poor survival in patients with hepatocellular carcinoma

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Abstract. Cystine/glutamic acid transporter (xCT) plays a role in tumor progression by regulating the redox status in several types of cancers. To demonstrate the importance of xCT expression for predicting the prognosis of hepatocellular carcinoma (HCC), we analyzed xCT gene expression in 130 paired HCC and non-cancerous tissues. xCT protein expression was confirmed using 7 HCC cell lines and samples from human subjects. xCT mRNA expression was detected in 34 (26%) tumor tissues. Expression of xCT was higher in HCC tissues compared to the corresponding normal tissues according to quantitative reverse transcriptase-polymerase chain reaction findings (P<0.0001). Patients in the group presenting with xCT mRNA expression showed poorer overall and disease-free survival than did those with an absence of xCT mRNA (P=0.0130 and 0.0416, respectively). xCT mRNA expression proved to be an independent factor for poor prognosis in a multivariate analysis of overall survival (hazard ratio, 1.68; 95% CI, 1.03-2.92). We observed xCT protein expression in both the HCC cell lines and in human tissue samples. In conclusion, the findings of the present study suggest that xCT is useful as a predictive marker for patient prognosis and that it may be a novel therapeutic target for HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common gastrointestinal malignancies and the leading cause of cancer-related death in East Asia and South Africa (1). At present, the first-line treatment for HCC is liver transplantation or surgical resection (2,3). However, the overall survival rate after curative therapy is not satisfactory due to the highly chemoresistant nature of this tumor and frequent intrahepatic recurrence. Identification of the genes responsible for the development and progression of HCC and comprehension of the clinical significance of these genes are critical for adequate treatment of HCC.

Glutathione (GSH) is a tripeptide thiol consisting of glutamate, cysteine and glycine, and it plays an important role in cellular defenses against oxidative stress and toxic compounds (4). Sustenance of GSH levels in cancer cells is essential for DNA synthesis, growth, multidrug resistance, maintenance of redox status and tumor survival (5-8). System x_c^-, consisting of cystine/glutamic acid transporter (xCT)/SLC7A11 and its chaperone CD98/4F2hc, functions as an exchange system for cysteine/glutamate (9). Since glutamate present in the extracellular medium can regulate cell signaling through its receptor, upregulation of x_c^- in tumor cells may also be associated with increased glutamate signaling in the tumor cells themselves or in adjacent cells (10,11). In contrast to normal cells, tumor cells are characterized by rapid growth and proliferation (5). This is partly due to the fact that tumor cells have cellular defenses against oxidative stress, facilitating the cell cycle and resistance to apoptosis. xCT is strongly associated with these systems and is therefore a potential therapeutic target (6,10). We identified a CD44 variant that regulates redox status by stabilizing xCT as a pivotal marker of gastric cancer (12), yet the clinical relevance of xCT in HCC has not yet been clarified.

We subsequently investigated the clinical importance of the xCT gene by analyzing 130 consecutive patients with HCC as well as several HCC cell lines. We suggest that xCT expression is a candidate marker of HCC prognosis.

Materials and methods

Clinical tissue samples. One hundred and thirty patients (106 men and 24 women) with HCC were enrolled and underwent curative first-line surgery at the Department of Gastroenterological Surgery, Kumamoto University Hospital, between 2005 and 2010. Specimens of primary HCC and

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Abbreviations: HCC, hepatocellular carcinoma; xCT, transporter responsible for amino acid transport system x_c^-; HPRT1, hypoxanthine phosphoribosyltransferase 1; ROS, reactive oxygen species

Key words: cystine/glutamic acid transporter, hepatocellular carcinoma, reactive oxygen species

adjacent normal liver tissues were procured from the patients after written informed consent was obtained. This study was approved by the Human Ethics Review Committee of the Graduate School of Life Sciences, Kumamoto University (Kumamoto, Japan).

Cell lines. The Li-7 cell line was purchased from Riken BioResource Center (Osaka, Japan) and was cultured in RPMI1640 medium (Wako, Osaka, Japan). The HepG2, PLC/PRF/5, HuH1, HuH-7, HLE and HLF cell lines were purchased from the Japanese Collection of Research Bioresources (Osaka, Japan) and the cells were cultured in Dulbecco's modified Eagle's medium (DMEM). All media were supplemented with 10% fetal bovine serum (FBS) with 100 units/ml penicillin and 100 μg/ml streptomycin. All cultures were maintained in a 5% CO_2/95% air humidified atmosphere at 37˚C.

RNA extraction and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Total RNA was obtained from the frozen tissue samples and cell lines using a mirVana microRNA isolation kit (Ambion) in accordance with the manufacturer's instructions. Reverse transcription was performed with 1.0 μg of total RNA as previously described (13). qRT-PCR was performed on a LightCycler 480 II using 2X PCR Master Mix and Universal Probe Library (all were from Roche Diagnostic, Tokyo, Japan). Primers were designed using the Roche webpage (http://app.roche-biochem.jp/) and the Universal Probe Library in accordance with the manufacturer's recommendations. The primers used were as follows: xCT forward, 5'-CCATGAACGGTGGTGTGTT-3' and reverse, 5'-GACCCTCTCGAGACGCAAC-3' and universal probe #80; HPRT forward, 5'-TGACCTTGATTTATTTTGCATACC-3' and reverse, 5'-CGAGCAAGACGTTCAGTCCT-3' and universal probe #73. HPRT, 18S ribosomal RNA, and GAPDH were tested as internal controls (14); HPRT proved to be the most suitable reference gene. For amplification, an initial denaturation at 95˚C for 10 min was followed by 15 sec at 95˚C, 15 sec at 60˚C and 13 sec at 72˚C. All experiments were performed twice to confirm reproducibility.

Western blotting. Cells were lysed in a cell lysis buffer containing 25 mM Tris (pH 7.4), 100 mM NaCl, and 1% Tween-20. Equal amounts of protein were loaded onto 10% gels and separated by SDS-PAGE. Resolved proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad), blocked with 5% low-fat dry milk in TBS-T (25 mM Tris pH 7.4, 125 mM NaCl, 0.4% Tween-20) for 1 h at room temperature and incubated with the primary antibody overnight at 4˚C. The primary antibody, mouse monoclonal xCT antibody (KE021; TransGene Inc., Hyogo, Japan), was used at a dilution of 1:1,000. Blots were extensively washed with TBS-T and incubated for 1 h at room temperature with HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:2,000 in TBS-T. The membranes were washed and visualized using a chemiluminescence detection reagent kit (ECL Plus; GE Healthcare Corp.).

Immunofluorescence staining. Approximately 6-μm cryostat sections of HCC were fixed for 10 min in 4˚C acetone and air-dried for 1 h. Incubated cells were washed with PBS twice and then fixed for 10 min in 4% paraformaldehyde. Subsequently, the sections/cells were washed twice in TBS and once in TBS/Tween-20 (0.05%) and then incubated with 3% bovine serum albumin (BSA) (Sigma, Japan) for 15 min to block nonspecific protein binding sites. They were subsequently incubated for 1 h at room temperature with the primary antibody in TBS containing 1% BSA. The primary antibody, mouse monoclonal xCT antibody, was used at a dilution of 1:25. Samples were incubated for 1 h at room temperature in the dark with the secondary antibody, goat anti-mouse IgG labeled with HiLyte Fluor™ 555 (Anaspec Inc., San Jose, CA, USA), in TBS containing 1% BSA. The mounting reagent was applied using ProLong Gold including 4',6-diamidino-2-phenylindole (Invitrogen, Japan). For negative controls, a mouse IgG (Dako Co., Japan) was used instead of the primary antibody. Images were obtained with a FV300 fluorescence microscope (Olympus, Japan).

Statistical analysis. Statistical analyses were performed using JMP ver. 8.0 (SAS Institute, Cary, NC, USA). Values are expressed as the means ± SD. Differences between groups were calculated by the Wilcoxon test. $P<0.05$ was defined as indicative of a statistically significant difference.

Results

Expression of xCT in clinical tissue specimens and clinicopathological characteristics. We performed a qRT-PCR analysis with the primary HCC specimens. xCT expression was calculated by xCT/hypoxanthine phosphoribosyltransferase 1 (HPRT1) expression. For the clinicopathological evaluation, patients were divided into 2 groups based on expression status. The expression value of xCT was detectable in 34 (26.1%) tumor tissues. Clinicopathological factors related to the xCT expression status of the 130 patients are summarized in Table I. xCT expression was not correlated with any of the clinicopathological factors.

Relationship between xCT expression and prognosis. xCT mRNA expression was higher in HCC tissues than in the corresponding normal tissues according to qRT-PCR analysis ($P<0.0001$, Fig. 1A). The relationship between each of the clinicopathological factors and prognosis was analyzed by univariate analyses (Table II). The data indicated that poor prognosis in HCC patients was correlated with a tumor diameter of >38 mm, multiple tumors, positive vascular invasion and positive xCT expression. Patients in the xCT mRNA present group had poorer overall and disease-free survival than did those in the xCT mRNA absent group ($P=0.0130$ and 0.0416, respectively) (Fig. 1B and C). The presence of xCT mRNA expression proved to be the only poor prognostic factor in a multivariate analysis of overall survival (Table III).

Expression of xCT protein. All of the cell lines expressed xCT mRNA (Fig. 2A), which corresponded with the expression of xCT protein (Fig. 2B). The representative immunohistochemical xCT staining patterns are shown in Fig. 2; membranous expression of xCT was confirmed in both the cell lines (Fig. 2C) and the tissues from HCC patients (Fig. 2D and E). One of the 8 randomly selected patients had xCT protein staining of the tumor cell membrane but not in the non-tumor

Table I. xCT expression and clinicopathological characteristics of the HCC patients.

Clinicopathological factors	n	xCT expression		P-value
		High	Low	
Age (years)				
<66	62	12	50	0.0921
≥66	68	22	46	
Gender				
Male	106	28	78	0.8867
Female	24	6	18	
AFP[b]				
<20	74	20	54	0.7945
≥20	56	14	42	
PIVKAII[a]				
<69	61	12	49	0.1138
≥69	69	22	47	
Tumor diameter (mm)[a]				
<38	63	12	51	0.0738
≥38	67	22	45	
Tumor number				
Solitary	97	25	72	0.8655
Multiple	33	9	24	
Differentiation				
Well/Mod	107	26	81	0.2993
Poor	23	8	15	
Vascular invasion[c]				
Negative	72	17	55	0.4623
Positive	58	17	41	
HCV-Ab				
Negative	72	21	51	0.3838
Positive	58	13	45	
HBs-Ag				
Negative	91	25	66	0.6013
Positive	39	9	30	

Well, well-differentiated hepatocellular carcinoma; Mod, moderately differentiated; Poor, poorly differentiated. [a]Cut-off value was defined as the median value. [b]Cut-off value was defined as the maximum normal value. [c]Pathological vascular invasion. AFP, α-fetoprotein; PIVKAII, protein induced by vitamin K absence/antagonist-II; HCV-Ab, hepatitis C virus antibody; HBs-Ag, hepatitis B virus surface antigen.

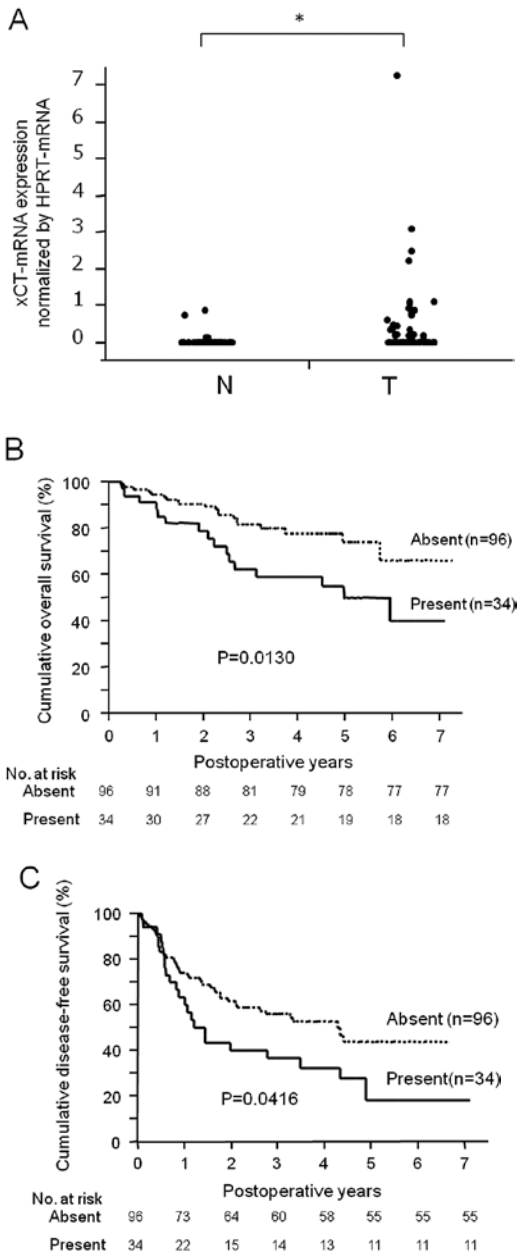

Figure 1. Clinical significance of xCT in hepatocellular carcinoma (HCC). (A) A quantitative RT-PCR analysis of xCT was performed in HCC (T) and paired normal tissue (N) specimens obtained from 130 patients. (B and C) Kaplan-Meier survival curves for patients with the presence or absence of xCT mRNA expression and its association with (B) cumulative overall survival and (C) cumulative disease-free survival.

tissue. The remaining 7 patients had no xCT expression in normal or cancerous tissues.

Discussion

The qRT-PCR results and multivariate analysis confirmed that the presence of xCT mRNA expression is an independent predictive factor for poor prognosis in HCC patients. Previous reports have shown that xCT expression plays a functional role in tumor progression. Inhibition of the transporter function with compounds such as sulfasalazine or (S)-4-carboxyphenylglycine suppresses tumor cell growth and invasion in glioma and HCC (5,11,15). Overexpression of xCT, which results in increased x_c^- activity, increases the levels and activity of the transcription factor AP-1 and promotes the cell cycle (16). In addition, xCT plays an important role in drug resistance in several types of tumors *in vitro* (17,18).

xCT mRNA expression was significantly higher in tumor tissues than in normal tissues in the HCC patients. Although xCT expression is not specific to tumor cells and has also been observed in normal cell types such as fibroblasts (19), monocytes (20) and macrophages (21), our results indicate that functional demand for xCT was higher in tumor tissues

Table II. Univariate analysis of the clinicopathological factors for overall survival in HCC patients.

Clinicopathological factors	n	Median survival (months)	P-value
Age[a] (years)			
<66	62	43.0	0.2836
≥66	68	37.3	
Gender			
Male	106	40.1	0.4509
Female	24	44.1	
AFP[b]			
<20	69	38.7	0.3001
≥20	63	41.1	
PIVKAII[a]			
<69	61	42.2	0.1658
≥69	69	38.2	
Tumor diameter[a] (mm)			
<38	63	45.2	<0.0001
≥38	67	33.1	
Tumor number			
Solitary	97	41.2	0.0014
Multiple	33	36.6	
Differentiation			
Well/Mod	107	41.7	0.0913
Poor	23	37.3	
Vascular invasion[c]			
Negative	72	42.7	0.0108
Positive	58	38.1	
HCV-Ab			
Negative	72	37.0	0.5459
Positive	58	43.8	
HBs-Ag			
Negative	91	42.3	0.3656
Positive	39	36.6	
xCT-mRNA expression			
Negative	96	41.1	0.0130
Positive	39	37.0	

Well, well-differentiated hepatocellular carcinoma; Mod, moderately differentiated; Poor, poorly differentiated. [a]Cut-off value was defined as the median value. [b]Cut-off value was defined as the maximum normal value. [c]Pathological vascular invasion. AFP, α-fetoprotein; PIVKAII, protein induced by vitamin K absence/antagonist-II; HCV-Ab, hepatitis C virus antibody; HBs-Ag, hepatitis B virus surface antigen.

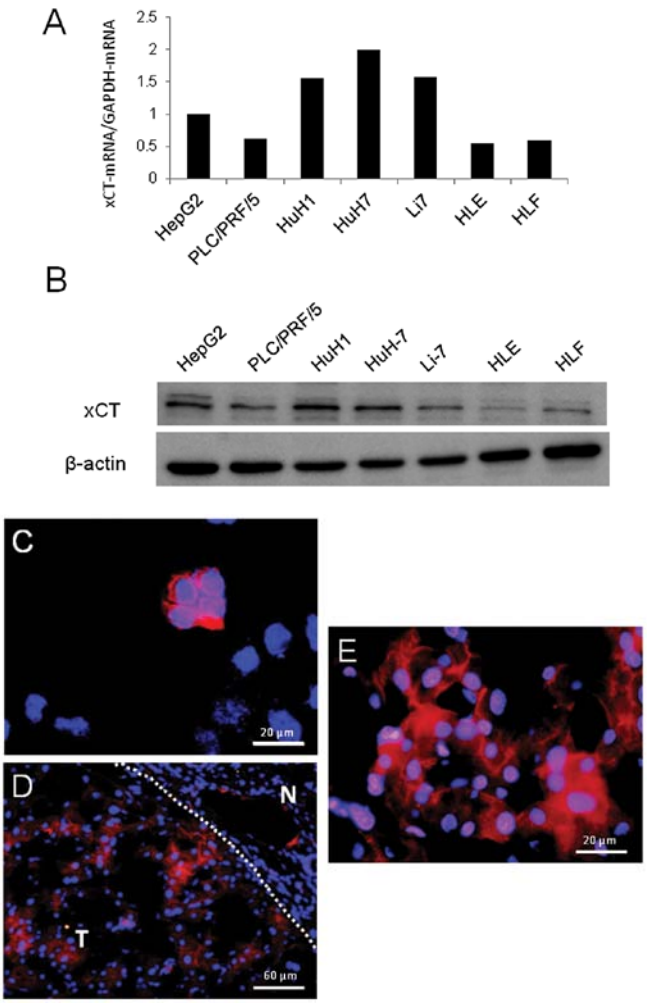

Figure 2. xCT protein expression in HCC cell lines and human HCC tissue samples. (A) xCT mRNA expression in 7 HCC cell lines (HepG2, PLC/PRF/5, HuH1, HuH-7, Li7, HLE and HLF) analyzed by quantitative RT-PCR. (B) xCT protein expression in the 7 HCC cell lines analyzed by western blotting. xCT expression in (C) HuH1 cells and in (D and E) human tissue specimens (sample no. 165) assessed by immunofluorescence staining. T, tumor tissue; N, normal tissue. Magnification, x200 in D, magnification, x600 in E.

Table III. Cox proportional hazards model for overall survival of the HCC patients.

Clinicopathological factor	P-value	Risk ratio (95% CI)
High xCT expression	0.0390	1.684 (1.026-2.915)
Multiple tumors	0.1034	1.558 (0.917-2.807)
Tumor diameter ≥38 mm	0.2398	1.303 (0.839-2.048)
Vascular invasion, positive	0.9867	1.004 (0.641-1.553)

CI, confidence interval.

than in normal tissues. However, we did not find any correlation between xCT mRNA expression and clinicopathological factors. Further studies with human subjects are required with the aim of determining the relevance of xCT expression to functional aspects of tumors, such as reactive oxygen species (ROS) level, expression of other amino transporters and chemosensitivity.

We attempted to confirm the localization of xCT in the tumor cell membrane since xCT is believed to function only in the form of a membranous protein and since few reports have found xCT expression on the tumor cell membrane (10,12). We confirmed the localization of the xCT protein using frozen

HCC human tissues as well as HCC cell lines. Positive xCT expression was much higher in the HCC cell lines than in the human tissue samples as the cell lines were incubated with cysteine and not cystine, resulting in xCT upregulation (22).

Sulfasalazine is a potent xCT inhibitor and has been used for the clinical treatment of inflammatory bowel disease and rheumatoid arthritis (23); it has been shown to arrest growth via cystine starvation in various types of cancer cells, including lymphoma, prostate cancer, HCC and breast cancer (6,15,24,25). Guo *et al* (15) demonstrated that xCT dysfunction increased intracellular ROS levels, resulting in the autophagic death of HCC cells. Only a few of our patients with HCC showed high xCT expression in this study and these patients could be candidates for xCT-targeted therapy.

In conclusion, the present study suggests that xCT is useful as a predictive marker for patient prognosis and may be a novel therapeutic target for HCC. We expect that the results of this study will aid in the selection of patients and in customizing therapy targeting xCT.

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