Original Article

Serum Galectin-1 Autoantibodies in Patients with Hepatocellular Carcinoma

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ABSTRACT

Background: Although galectin-1 expression has been investigated, the clinicopathological significance of serum galectin-1 autoantibodies has not been evaluated in patients with hepatocellular carcinoma (HCC). This study investigated the clinicopathological significance of serum galectin-1 autoantibodies in patients with HCC.

Methods: Serum samples from 117 patients with HCC and 72 healthy individuals were analyzed by using an enzyme-linked immunosorbent assay system specifically developed to detect serum galectin-1 autoantibodies. The optical density cutoff value was set at 0.162 (the mean value for the controls plus 3 standard deviation). In patients positive for serum galectin-1 autoantibodies, clinicopathological characteristics were analyzed, including tumor stage and positivity rates for the conventional tumor markers alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist-II (PIVKA-II).

Results: The overall positivity rate for serum galectin-1 autoantibodies was 25%, which was lower than the positivity rate of 48% for AFP and 89% for PIVKA-II. No clinicopathological characteristic was associated with serum galectin-1 autoantibodies status.

Conclusions: Serum galectin-1 autoantibodies were present in patients with HCC, and serum galectin-1 autoantibodies positivity might be associated with HCC tumor progression. Although the differences between subgroups were not statistically significant, the combination of serum galectin-1 autoantibodies and a conventional tumor marker such as AFP and PIVKA-II might improve the rate of HCC detection. (Clinical trial registration number: UMIN 000014530)

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KEYWORDS: galectin-1, hepatocellular carcinoma (HCC), enzyme-linked immunosorbent assay

Hepatocellular carcinoma (HCC) accounts for greater than 90% of primary liver cancers and is a major global health concern.[1)] Despite the development of less invasive surgical treatments[2)] and new drugs, HCC remains the fourth and ninth leading cause of morbidity in Japanese men and women, respectively. Inadequate screening is associated with presentation with advanced tumors, poor prognosis, and high mortality rates. Early detection of

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HCC in high-risk patients is essential in improving prognosis. Alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist-II (PIVKA-II) are useful serum markers for detecting HCC.[3] However, these conventional serum markers frequently yield negative test results in the early stages of HCC. Recently, some immunoglobulin G (IgG) autoantibodies were found to respond to tumor-associated antigens in patient sera, even during early carcinogenesis. Using IgG autoantibodies such as NY-ESO-1, p53, HRAS1, and RalA, researchers were able to satisfactorily differentiate between HCC and healthy control sera.[4]

Galectin-1 (Gal-1), an endogenous lectin found at sites of immune privilege, has a critical role in regulating the immune response, and Gal-1 overexpression is clinically important in several benign diseases. Recently, Gal-1 overexpression has been reported in tumor tissues such as HCC.[5–7] Although several serum IgG autoantibodies have been investigated in patients with HCC, serum Gal-1 autoantibodies (s-Gal-1-Abs) have not been investigated in this population. Therefore, the aim of this study was to analyze s-Gal-1-Abs and evaluate the clinicopathological significance of s-Gal-1-Abs in patients with HCC.

Methods

Sera from patients and healthy donors

Sera were obtained from 117 patients before treatment for HCC: 50 samples were obtained from Biobank (Biobank Japan, Tokyo, Japan) and 67 samples were obtained from Chiba Cancer Center and Toho University Omori Medical Center. Samples obtained from Biobank were anonymous and had no information on patient sex, age, or type of viral infection. The control group comprised 72 samples obtained from healthy donors. The TNM stage of HCC was classified on the basis of the General Rules for the Clinical and Pathological Study of Primary Liver Cancer (5th Edition).[8] Among the 117 patients, 25 had stage I disease, 47 had stage II disease, 33 had stage III disease, and 12 had stage IV disease. Among the 67 patients at Chiba Cancer Center or Toho University, 48 were men and 19 were women (median age, 68 years; range, 52–86 years). Nine patients were hepatitis B virus (HBV)–positive and 35 were hepatitis C virus (HCV)–positive. This study was approved by the institutional review boards of the Chiba Cancer Center (#20-1) and Toho University School of Medicine (# 22-112, #22-047, #24-045). Written informed consent was obtained from all subjects. This clinical study was registered in the UMIN Clinical Trials Registry (#UMIN 000014530).

Purification of recombinant Gal-1 protein

For the expression and purification of recombinant protein, the full-length complementary deoxyribonucleic acid (cDNA) of Gal-1 (GenBank accession number: NM 002305) was amplified using polymerase chain reaction. The amplified gene was inserted between *Bam*HI and *Xho*I in a pET 28a(+) plasmid (Novagen®, Darmstadt, Germany) expressed as a C-terminal 6× histidine tag. This recombinant protein was expressed in *Escherichia coli* (*E. coli*) BL21-CodonPlus (DE3)-RIL (Stratagene, La Jolla, CA, USA) and was dissolved in phosphate-buffered saline (PBS). Gal-1 extract was applied to TALON® Metal Affinity Resin (Clontech Laboratories Inc., Mountain View, CA, USA), and purified Gal-1 recombinant protein was eluted using 20–500 mM imidazole in PBS. The expression and purity of recombinant protein was determined by performing 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis. DNA sequencing analysis of the constructed plasmid confirmed that this inserted gene was correct.

Enzyme-linked immunosorbent assay for detection of s-Gal-1-Abs and other conventional tumor markers

Serum samples were analyzed using an enzyme-linked immunosorbent assay, as previously described.[9, 10] Purified recombinant proteins were coated onto Maxisorp™ 96-well microtiter plates (Nalge Nunc International, Rochester, NY, USA). Gal-1 was diluted in PBS to a final concentration of 2.0 μg/ml and added to the plates (100 μl/well), which were then incubated overnight at 4℃. PBS was used as the control. After two washes with PBS, the proteins were blocked using 200 μl of PBS containing 1% bovine serum albumin and 5% sucrose at room temperature for 3 h. All human sera were diluted (1 : 100) in PBS containing 0.15% Tween 20, 1% casein, and 0.2 mg/ml *E. coli* extract. Then, 100 μl of the diluted sera was added to each Gal-1− or PBS-filled well and incubated at room temperature during centrifugation at 250 rpm for 60 min. After washing with PBS containing 0.05% Tween®-20 (PBST) four times, 100 μl of horseradish peroxidase − conjugated antihuman IgG (1 : 5000; Medical & Biological Laboratories [MBL] Co., Ltd., Nagoya, Japan) diluted in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 135 mM NaCl, 1% bovine serum albumin, and 0.1% hydroxyphenylacetic acid was added to each well as a secondary antibody and incubated at room temperature with centrifugation at 250 rpm for 60 min. The wells were

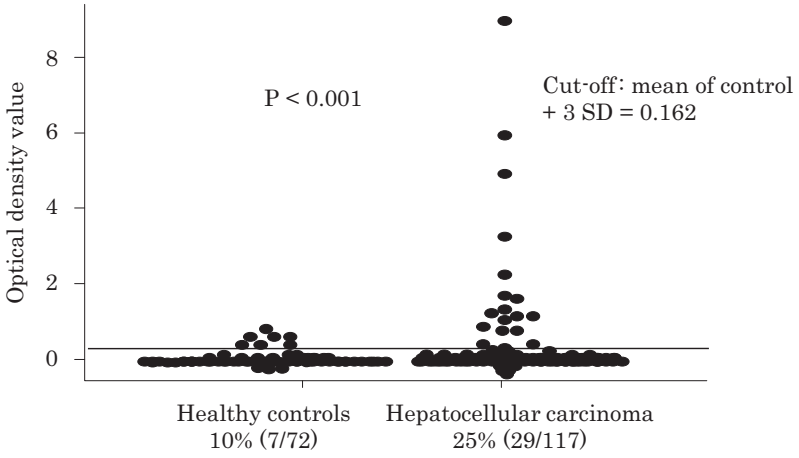

Fig. 1 Optical density values for serum galectin-1 autoantibody titers in patients with hepatocellular carcinoma and healthy controls.
SD: standard deviation

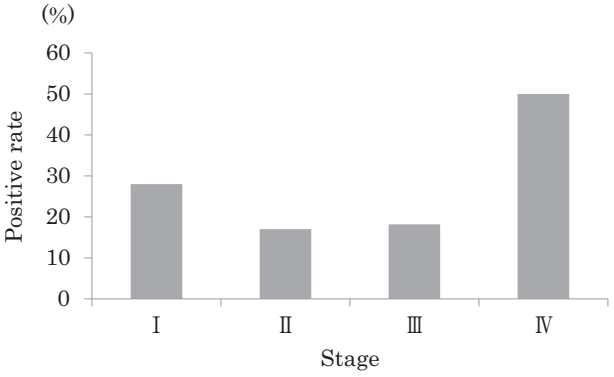

Fig. 2 Positivity rates for serum galectin-1 autoantibodies in patients with hepatocellular carcinoma, according to tumor stage.

washed four times with PBST buffer, and autoantibodies were detected by the addition of 100 µl of 3, 3', 5, 5'-tetramethylbenzidine substrate. After incubation at room temperature for 30 min, the reaction was stopped by the addition of 0.25 N sulfuric acid (100 µl/well). Absorbance was measured at 450 nm using a SUNRISE™ Microplate Reader (Tecan Japan Co., Ltd., Kawasaki, Japan). Gal-1 signals were evaluated by calculating the difference in absorbance between the wells containing Gal-1 and PBS. Serum AFP and PIVKA-II were also evaluated, as previously described.[11, 12]

Statistical analysis

Fisher's exact (two-sided) probability test and the Mann-Whitney *U* test were used to analyze differences between groups. All statistical analyses were performed using EZR (Easy R),[13] which is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0). More precisely, it is a modified version of R commander (version 1.6-3) and is designed to add statistical functions frequently used in biostatistics. A p value of less than 0.05 was considered to indicate statistical significance.

Results

Serum titers of s-Gal-1-Abs

Optical density values (mean ± 3 standard deviation [SD]) for s-Gal-1-Abs were significantly higher in patients with HCC (0.388 ± 1.165) than in healthy controls (0.032 ± 0.043; $p < 0.001$) (Fig. 1). The s-Gal-1-Abs titers were divided into two groups: negative optical density values (< 0.162; the mean + 3 SD of the values in healthy controls) and positive values (> 0.162).

Clinicopathological characteristics, conventional serum markers, and s-Gal-1-Abs status

Clinicopathological characteristics and conventional tumor markers were analyzed in 67 of the 117 patients. The s-Gal-1-Abs positivity rate was higher in patients with stage III/IV tumors than in those with stage I/II tumors (28% in stage I, 17% in stage II, 19% in stage III, and 50% in stage IV) (Fig. 2). Sex, age, HBV infection status, HCV infection status, and TNM stage did not significantly differ between s-Gal-1−positive and −negative patients. The associations between AFP, PIVKA-II, and s-Gal-1-Abs were also analyzed in these 67 individuals (Table 1); however, s-Gal-1-Abs positivity was not associated with AFP positivity or PIVKA-II positivity (Table 1). Because several previous studies reported that Gal-1 overexpression was signifi-

Table 1 Clinicopathological characteristics of patients with serum galectin-1 antibodies.

Variable		Total (n = 67)	Galectin-1 antibodies (+) (n = 19)	P value
Sex	Male	48	17	0.07
	Female	19	2	
Age, years	65≤	47	13	1
	65>	20	6	
HBV infection status	Negative	55	17	0.43
	Positive	9	1	
	Unknown	3	1	
HCV infection status	Negative	30	6	0.27
	Positive	35	12	
	Unknown	2	1	
TNM stage	I + II	31	8	0.79
	III + IV	36	11	
AFP	Negative	32	8	0.59
	Positive	32	11	
	Unknown	3	0	
PIVKA-II	Negative	18	6	0.77
	Positive	45	13	
	Unknown	4	0	

HBV: hepatitis B virus, HCV: hepatitis C virus, AFP: alpha-fetoprotein, PIVKA-II: protein induced by vitamin K absence or antagonist-II

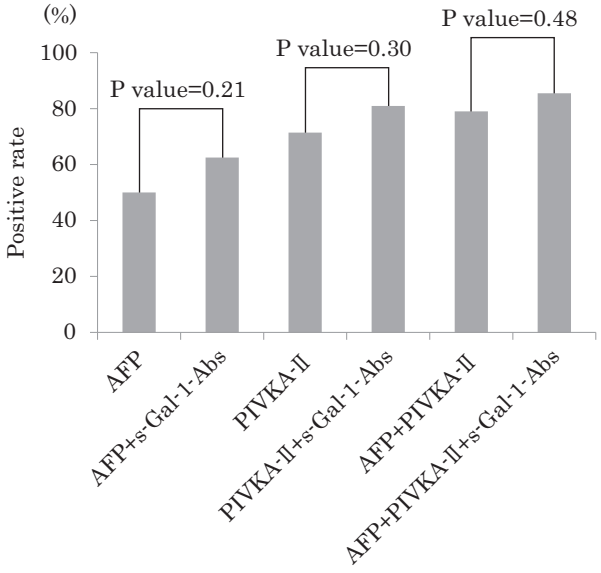

Fig. 3 Positivity rates for conventional tumor markers alone and in combination with serum galectin-1 autoantibodies.
AFP: alpha-fetoprotein, s-Gal-1-Abs: serum galectin-1 autoantibodies, PIVKA-II: protein induced by vitamin K absence or antagonist-II

cantly associated with tumor aggressiveness,[5–7] s-Gal-1-Abs may also reflect tumor progression. However, because only four of the present patients were s-Gal-1-Abs–positive, AFP–negative, and PIVKA-II–negative, we were unable to observe any specific clinicopathological characters in those patients.

s-Gal-1-Abs positivity in patients with HCC

The positivity rate for s-Gal-1-Abs (25%) was lower than those for AFP (50%) and PIVKA-II (71%). Although these differences were not statistically significant, assays that combined s-Gal-1-Abs with conventional tumor marker had higher rates of positive results. The positivity rate for the combination of AFP and/or s-Gal-1-Abs was higher than that for AFP alone (62% vs. 50%, respectively; p = 0.21). In addition, the positivity rate for the combination of PIVKA-II and/or s-Gal-1-Abs was higher than that for PIVKA-II alone (81% vs. 71%, respectively; p = 0.30) (Fig. 3).

Discussion

Although s-Gal-1-Abs have been analyzed in systemic lupus erythematosus (SLE) and rheumatoid arthritis,[14, 15] these antibodies have not been analyzed in cancer patients. In this report, we assayed s-Gal-1-Abs in patients with HCC. s-Gal-1 antibody status was not associated with AFP or PIVKA-II status. However, because s-Gal-1-Abs positivity was independent of conventional tumor markers, a combination of s-Gal-1-Abs and conventional tumor

markers might increase sensitivity for the detection of HCC.

Gal-1 upregulation was associated with increased HCC growth and metastasis in the liver tumor microenvironment.[5] A correlation between Gal-1 expression and HCC cell migration and invasion was also reported.[6] Gal-1 is a multifunctional protein involved in various aspects of tumorigenesis (cell–extracellular matrix and cell–cell interactions, cell migration, angiogenesis, and tumor–immune escape),[7] and expression of Gal-1 correlates with tumor aggressiveness.[16] Recurrence-free survival rates need to be compared between sero–positive and –negative patients, to confirm the predictive value of s-Gal-1-Abs for malignant potential and tumor recurrence. Because only four of the present patients were s-Gal-1-Abs–positive, AFP–negative and PIVKA-II–negative, we were unable to observe any specific clinicopathological characters in those patients. Because other types of tumors overexpress Gal-1, s-Gal-1-Abs might also be detectable in patients with other cancers. Future studies should investigate the clinical significance of s-Gal-1-Abs in patients with other cancers.

Montiel reported that Gal-1 had a role in modulating the immune system and that the presence of s-Gal-1-Abs may contribute to immune dysregulation in SLE.[15] SLE is an autoimmune disease characterized by T and B lymphocyte dysfunction and excessive production of various autoantibodies. A possible mechanism for SLE pathogenesis is the accumulation of autoantigens released by apoptotic cells, which causes abnormal activation of the immune system and increased production of pathogenic autoantibodies.[17] People with SLE in the United Kingdom had a greater comorbidity burden and were more likely to develop cancer than people of the same age and sex.[18] This tendency to develop malignant tumors could reflect immune dysregulation. Unfortunately, we did not assess the immunological characteristics of the present patients.

In conclusion, we found that s-Gal-1-Abs were present in patients with HCC. The rate of s-Gal-1-Abs positivity might be associated with HCC tumor progression. The combination of s-Gal-1-Abs with a conventional tumor marker (AFP and PIVKA-II) improved the positivity rate for detection of HCC. Because s-Gal-1-Abs might be associated with immunological disorders, a larger-scale study is necessary in order to confirm the clinical significance of these antibodies in tumor immunology.

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Disclosure Statement: Akiko Kuwajima is an employee of Medical & Biological Laboratories Co., Ltd., Nagoya, Japan. The other authors have no conflict of interest.

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