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ORIGINAL ARTICLE

Clusterin expression in nontumor tissue in patients with resectable hepatocellular carcinoma related with postresectional survival

Po-Chung Kuo[a], Ivy Yenwen Chau[a], Anna Fen-Yau Li[b], Yat-Pang Chau[c,*], Cheng-Yuan Hsia[a], Gar-Yang Chau[a,*]

[a]Department of Surgery, Taipei Veterans General Hospital, Taipei, Taiwan, ROC; [b]Department of Pathology, Taipei Veterans General Hospital, Taipei, Taiwan, ROC; [c]Department of Medicine, Mackay Medical College, New Taipei City, Taiwan, ROC

Abstract

Background: Surgical resection offers an effective treatment for patients with hepatocellular carcinoma (HCC); however, it has high tumor recurrence rate. Clusterin is a highly conserved glycoprotein that enhances cell aggregation in vitro. It is upregulated in several types of cancers such as breast, ovarian, colon, prostate and kidney cancers, and HCC. Clusterin overexpression is correlated with tumor metastasis. We evaluated the significance of clusterin expression levels in serum and resected tissues of patients with HCC.

Methods: Serum, resected tumor tissue, and nontumor tissue were collected from 140 patients with HCC undergoing hepatic resection. Serum clusterin levels were determined by enzyme-linked immunosorbent assay. Clusterin expression in resected tissue was evaluated by immunohistochemistry. Median follow-up time was 57.8 months.

Results: Mean serum clusterin levels were found to be 130.0 ± 58.7 μg/mL (range, 10.1-366.6 μg/mL). Serum clusterin levels were independent of tumor stage and deterioration of liver function in patients. No significant difference was observed in the survival of patients with high (>130.0 ± 58.7 μg/mL) or low (≤130.0 ± 58.7 μg/mL) serum clusterin level. Clusterin was expressed in HCC tissues of 76 patients (54.3%) and nontumor liver tissues of 53 patients (37.9%). No significant difference was observed in the survival of patients with positive or negative clusterin expression in HCC tissues. In nontumor tissues, patients with positive clusterin expression were observed to have low postoperative disease-free survival rate (p = 0.001) compared to patients with negative clusterin expression. Multivariate analysis showed that tumor with macrovascular/microvascular invasion and clusterin expression in nontumor tissues are independent prognostic factors following hepatic resection.

Conclusion: In HCC, clusterin expression in nontumor tissue shows worse prognosis after hepatic resection. Clusterin can be a prognostic marker for patients with postresection HCC.

Keywords: Clusterin; Hepatocellular carcinoma; Outcome predictor

1. INTRODUCTION

Surgical resection offers effective treatment for patients with hepatocellular carcinoma (HCC); however, it is associated with high postoperative tumor recurrence rate.[1] Vascular invasion is shown to be the most important factor associated with early tumor recurrence.[2] However, normal functioning of the liver has also been reported to contribute in HCC recurrence.[3]

Clusterin, initially isolated from the rat testis, is a highly conserved glycoprotein that enhances cell aggregation in vitro.[4] Clusterin has been reported to contribute in tissue remodeling, cell adhesion, cell apoptosis, cell proliferation, cell senescence, membrane recycling, lipid transportation, complement inhibition, and sperm maturation.[5] Furthermore, clusterin has been shown to be upregulated in tumor pathogenesis and progression. Upregulated clusterin expression has been reported in many cancers such as breast, ovarian, colon, prostate and kidney cancers, and HCC.[6–11] In addition, many studies have shown that clusterin is downregulated in nonmelanoma skin cancer, esophageal cell carcinoma, and prostatic carcinoma.[12–14] Thus, previous investigations suggest that clusterin expression, whether upregulated or downregulated, might play a crucial role in tumorigenesis.[15]

The objective of our study was to investigate the significance of clusterin expression level in serum and resected tissue of 140 patients with HCC.

2. METHODS

2.1. Patients and collection of clinicopathological data

Serum, resected tumor tissue, and nontumor liver tissue were obtained from 140 patients with HCC who underwent curative hepatic resection between May 1998 and February 2000 at Taipei Veterans General Hospital (Taipei, Taiwan). Patients who had received any preoperative treatment such as chemotherapy, ethanol

*Address Correspondence: Dr. Gar-Yang Chau, Division of General Surgery, Department of Surgery, Taipei Veterans General Hospital, 201, Section 2, Shi-Pai Road, Taipei 112, Taiwan, ROC. E-mail address: gychau@vghtpe.gov.tw (G.-Y Chau); Dr. Yat-Pang Chau, Department of Medicine, Mackay Medical College, 46, Section 3, Zhongzheng Road, Sanzhi Dist., New Taipei City 252, Taiwan, ROC. E-mail address: leonchau@mmc.edu.tw (Y.-P Chau).

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injection, or transarterial chemoembolization were not included in the study. HCC diagnosis was confirmed by histological examination of the surgically resected specimens. Curative resection was defined as complete removal of the tumor macroscopically with a negative surgical margin on histological examination. The study was approved by Committee for the Conduct of Human Research of the Taipei Veterans General Hospital. Informed consent was obtained from all the patients prior to the study. Blood samples were collected from all patients following an overnight fast and prior to operation. Serum was isolated immediately and stored at –20°C. Each sample underwent only a single freeze-thaw cycle. Furthermore, tumor specimens were obtained immediately following surgical resection. The liver tissues were snap frozen in liquid nitrogen and stored at –70°C. Preoperative clinical and laboratory data, histological analysis of the resected specimens, and postoperative survival data were prospectively obtained. Detailed clinicopathological features including routine liver biochemistry, hepatitis markers, serum α-fetoprotein (AFP) level, tumor size, tumor number, and vascular invasion were documented. Histological assessment was based on reviewing the hematoxylin and eosin-stained sections. HCC tissues were categorized as per American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) 7th edition tumor, node, and metastasis (TNM) staging system. We correlated clusterin expression in HCC specimens and in serum with important clinicopathological parameters including age, sex, status of anti-hepatitis C virus (HCV), status of hepatitis B surface antigen (HBsAg), serum AFP level, tumor size, number of tumors, indocyanine green retention rate at 15 minutes (ICG-15) level, serum albumin level, serum alanine aminotransferase level, macroscopic/microscopic vascular invasion, and liver cirrhosis.

2.2. Enzyme-linked immunosorbent assay for clusterin expression in serum

Serum clusterin levels were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kit (BioVendor Laboratory Medicine Inc, Modrice, Czech Republic) following the manufacturer's instructions. All samples and standards were evaluated in duplicates, and the mean values were calculated. Briefly, samples and reference standard dilutions were pipetted in 96-well plates. After incubation at room temperature for 2 hours, the plates were washed and incubated with clusterin conjugate. Subsequently, the plates were incubated for 2 hours at room temperature followed by washing. Substrate solution was then added to each well and incubated at room temperature for 30 minutes. The reaction was stopped by adding stop solution and the plates were read at 450 nm by Spectra Max 250 ELISA plate reader (Molecular Devices., Hampton, New Hampshire, USA). Clusterin concentration was expressed in μg/mL and evaluated by comparing with the reference standards.

2.3. Immunohistochemistry

Samples were fixed in 4% paraformaldehyde at 4°C overnight and embedded in wax using automatic embedding machine. Further, the samples were dehydrated through series of ethanol gradients (70%, 80%, 90%, and 100%) followed by clearing with chloroform and then embedded in paraffin. Five-micron sections were sliced and immunostaining was performed using Universal DAKO LSAB2 system (DAKO, Carpinteria, CA, USA) according to manufacturer's instructions. Briefly, the rehydrated tissue sections were treated with sodium citrate buffer (10 mM, pH 6) in a microwave, incubated with 3.0% H_2O_2 for 10 minutes, and soaked with blocking solution for additional 10 minutes. The tissue sections were then incubated overnight with monoclonal antibody to clusterin (clone B-5, 1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C in a moist chamber. Further, tissue section slides were washed in phosphate buffered saline and incubated with biotin-labeled secondary antibody for 10 minutes followed by treatment with streptavidin horseradish peroxidase conjugate for additional 10 minutes. Stains were developed using 3,3'-diaminobenzidine substrate for 10 minutes and then counterstained using Mayer's hematoxylin for 10 minutes (MUTO Chemicals CO, Tokyo, Japan). The slides were mounted and evaluated under a microscope. Consecutively, negative controls were performed without primary antibody. Clusterin expression was evaluated as follows: negative (–) for weak or no staining, (+) for positive expression observed in <10% of the tumor cells, and positive overexpression in >10% of the tumor cells were assigned moderate (2+) to strong (3+) staining.

2.4. Statistical analysis

Data are presented as mean ± SD except where indicated. Statistical difference between means of different treatment groups was determined using either one-way analysis of variance or Student's *t* test. Statistical analysis was performed to evaluate disease-free survival

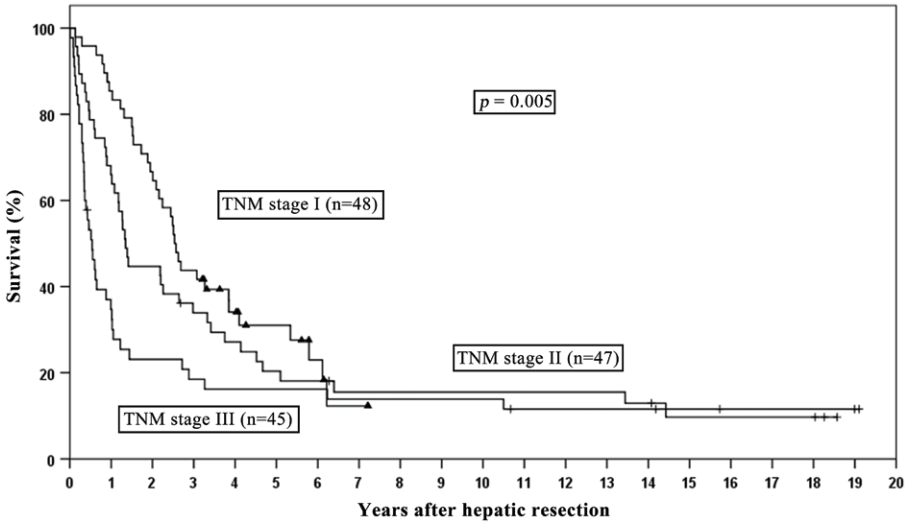

Fig. 1 Survival curves for patients with HCC stratified into TNM stage I, stage II, and stage III. Survival rate was significantly and inversely correlated with TNM staging ($p = 0.005$). HCC, hepatocellular carcinoma; TNM, tumor-node-metastasis.

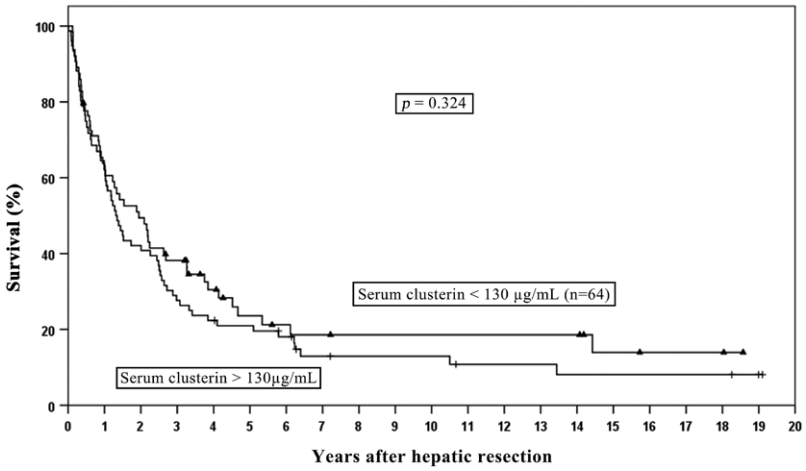

Fig. 2 Survival curves for patients with HCC stratified according to the preoperative serum clusterin level. No survival difference was observed on comparing patients with high serum clusterin level (>130.0 µg/mL) or low serum level (≤130.0 µg/mL) (p = 0.324). HCC, hepatocellular carcinoma.

rate. Disease-free survival was defined as the time from surgery to diagnosis of local recurrence, metastasis, or death. Survival rates were evaluated using Kaplan-Meier method, and survival curves were compared using log-rank test. Multivariate analysis was performed using Cox proportional hazards model to determine independent factors related to survival. The results are expressed as risk ratios with 95% CIs. Statistical Package for the Social Sciences (SPSS, IBM *SPSS Statistics 20.0 for Windows*) version 20.0 was used for statistical analysis. $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Clinical parameters

Mean age of the patients included in the study was found to be 62.3 ± 13.6 years, and 113 patients (80.7%) were male. Furthermore, 73 patients (52.1%) were observed to be HBsAg positive, 34 (24.3%) were anti-HCV positive, and 22 (15.7%) had a family history of HCC. Mean tumor size was found to be 5.3 ± 3.7 cm (range, 0.8-20.0 cm). Eighty-eight patients (62.9%) were diagnosed with solitary HCC, while 29 patients (20.7%) were detected with macrovascular invasion and 88 patients (62.9%) showed microvascular invasion. As per 7th edition of AJCC/UICC TNM staging system, patients were divided into three TNM classified stages: stage I (n = 48), stage II (n = 47), and stage III (n = 45). The disease-free survival rates of patients belonging to all the three stages are shown in Figure 1. Fifty-eight patients (41.1%) were found to be cirrhotic. Thus, majority of patients were diagnosed for chronic liver disease with abnormal liver function and mean serum ICG-15 of 14.5 ± 10.8% (normal 0%-10%).

3.2. Serum clusterin level and postoperative survival

Preoperative serum clusterin level was found to be 130.0 ± 58.7 µg/mL (range, 10.1 -366.6 µg/mL). Results revealed that serum clusterin levels did not correlate with the tumor stages and the

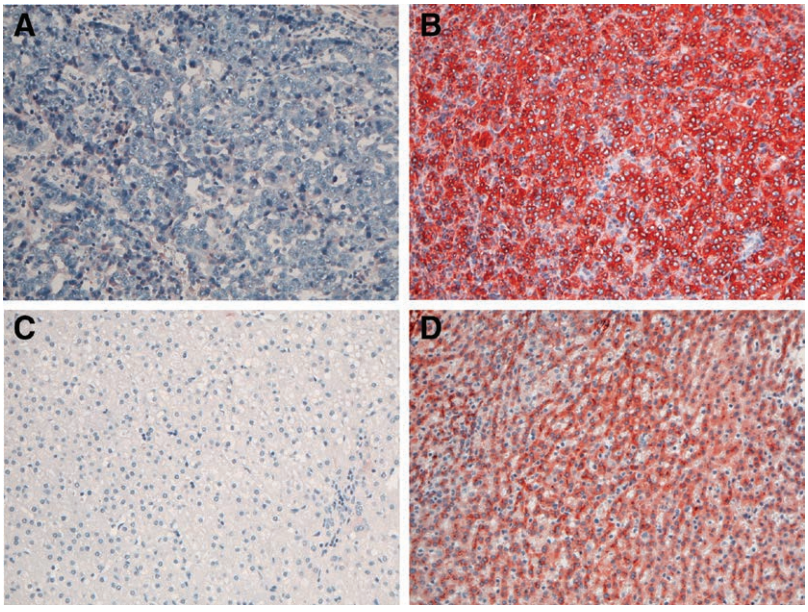

Fig. 3 Immunohistochemical detection of clusterin protein in (A) HCC, negative; (B) HCC, positive; (C) paired nontumor tissues, negative; and (D) paired nontumor tissues, positive; in TNM stage I. Bar = 80 µm. HCC, hepatocellular carcinoma; TNM, tumor-node-metastasis.

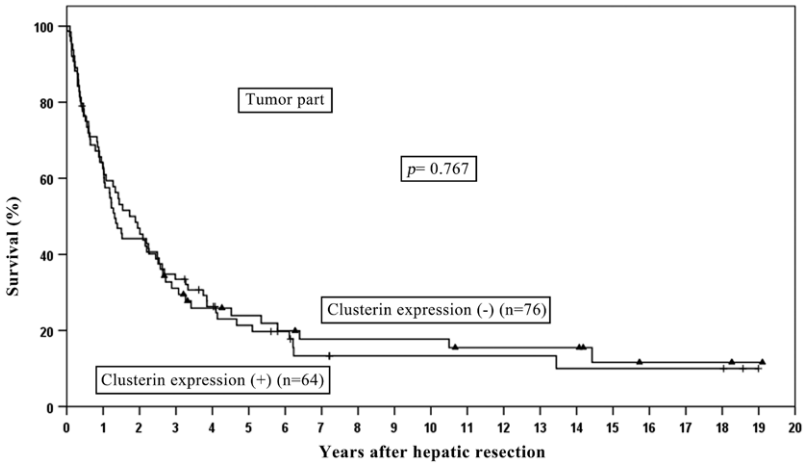

Fig. 4 Comparison of survival curves for patients based on clusterin expression in HCC tissues. No survival difference was observed on comparing patients with positive or negative expression (p = 0.767). HCC, hepatocellular carcinoma.

degree of deterioration of functional liver. Mean follow-up time of patients was 57.8 months (range, 1.4-272 months). Univariate analysis revealed no survival difference on comparing patients with preoperative high serum clusterin levels (≥130.0 ± 58.7 μg/mL, n = 76) to patients with low serum clusterin levels (<130.0 ± 58.7 μg/mL, n = 64) (p = 0.324) (Fig. 2).

3.3. Immunohistochemistry staining of clusterin in HCC and nontumor liver tissue

Clusterin expression was detected in HCC tissues of 76 patients (54.3%) and in nontumor liver tissues of 53 patients (37.9%) (Fig. 3A–D). Similar clusterin expression patterns were found in patients with TNM stage I (n = 48, 58.3%), stage II (n = 47, 57.4%), and stage III (n = 45, 46.7%) HCC (p = 0.459). No survival difference was observed between patients with positive or negative clusterin expression in HCC tissue (p = 0.767) (Fig. 4). Patients with positive clusterin expression in nontumor tissue were found to have low postoperative disease-free survival rate (p = 0.001) as shown in Figure 5. Further, clusterin expression in nontumor tissue was associated with clusterin expression in resected tumors using statistical survival analysis. The patients were divided into four different groups: group 1: clusterin$^+$/nontumor$^+$ (n = 40); group 2: tumor$^+$/nontumor$^-$ (n = 36); group 3: tumor$^-$/nontumor$^+$ (n = 13); and group 4: tumor$^-$/nontumor$^-$ (n = 51). We found that group 2 patients had better survival rate than group 1 (p = 0.047) and group 3 (p = 0.002) patients. Similarly, group 4 patients were found to have better survival than group 1 (p = 0.043) and group 3 (p = 0.001) patients. However, no survival difference was observed between group 1 and group 3 and between group 2 and group 4. Furthermore, the tumor recurrence rate was found to be significantly higher in patients with positive clusterin expression in nontumor tissue (77.4%) compared to patients with negative clusterin expression (60.9%) (p = 0.045). No significant difference was observed in tumor recurrence rates between groups with positive (71.1%) or negative (62.5%) clusterin expression in HCC tissue (p = 0.283).

Comparisons of clinicopathological characteristics of HCC patients based on clusterin expression in nontumor tissues are listed in Table 1.

Univariate analysis revealed that significant factors related with survival rate were serum albumin ≤4.0 g/dL, multiple tumors, occurrence of macrovascular/microvascular invasion, TNM stage, and positive expression of clusterin in nontumor tissue (Table 2). Multivariate analysis using Cox proportional

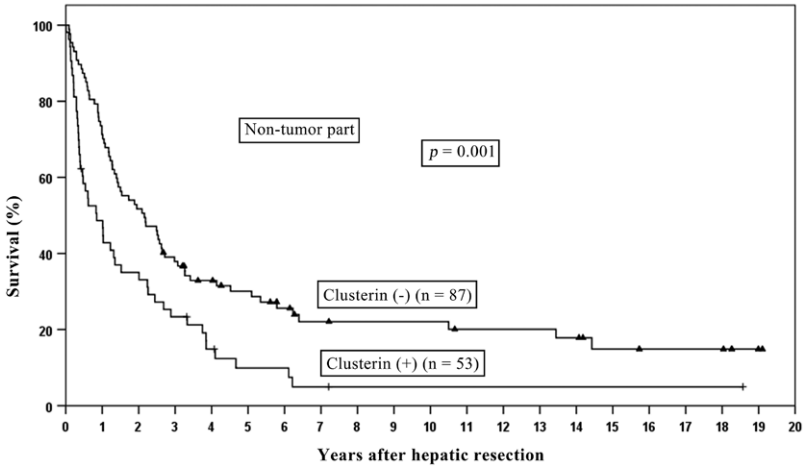

Fig. 5 Comparison of survival curves for patients based on clusterin expression in nontumor tissues. Patients with positive clusterin expression showed worse postoperative survival (p = 0.001).

Table 1

Comparison of clinic-pathological characteristics of HCC patients according to clusterin expression in nontumor part

Variables	Clusterin (–) (n = 87)	Clusterin (+) (n = 53)	*p*
Age, y	64 ± 13	59 ± 14	0.048[a]
Male, n (%)	70 (80.5)	43 (81.1)	0.922
With family history of HCC, n (%)	13 (14.9)	9 (17.0)	0.705
HBsAg positive, n (%)	41 (47.1)	32 (60.4)	0.128
Anti-HCV positive, n (%)	22 (25.3)	12 (22.6)	0.723
AFP level, ng/mL	2154 ± 7855	1784 ± 10505	0.814
Albumin, g/dL	4.0 ± 0.4	3.9 ± 0.4	0.018[a]
ALT, U/dL	52 ± 53	64 ± 64	0.237
Tumor size, cm	5.2 ± 3.8	5.6 ± 3.5	0.479
Multiple tumor, n (%)	35 (40.2)	17 (32.1)	0.333
Macrovascular invasion, n (%)	15 (17.2)	14 (26.4)	0.194
Microvascular invasion, n (%)	52 (59.8)	36 (67.9)	0.333
TNM stage, n (%)			
I	31 (35.6)	17 (32.1)	0.762
II	30 (34.5)	17 (32.1)	
III	26 (29.9)	19 (35.8)	
Liver cirrhosis, n (%)	34 (39.1)	24 (45.3)	0.470
Surgical margin, cm	0.9 ± 0.9	0.8 ± 1.0	0.489

[a]Statistically significance.

AFP = α-fetoprotein level; ALT = alanine aminotransferase (normal range 0-40 U/L); anti-HCV = anti-hepatitis C virus antibody; HBsAg = hepatitis B surface antigen; HCC = hepatocellular carcinoma; TNM = tumor-node-metastasis.

hazards model revealed that occurrence of macrovascular/microvascular invasion and positive expression of clusterin in nontumor sections of liver tissue were independent factors that were related to survival rate (Table 3).

4. DISCUSSION

In this study, we evaluated clusterin concentrations in the serum of patients with HCC before tumor resection and in the resected tissues, including HCC tissue as well as adjacent nontumor tissue. Our results suggested that preoperative serum clusterin levels and protein expression in HCC tissues are not related to the patient outcome after HCC resection. However, we found that patients with positive expression of clusterin in nontumor tissue show worse prognosis compared to patients with negative clusterin expression in nontumor tissue.

Recently, Nafee et al[16] showed that serum clusterin levels were significantly increased in patients with HCC. Further, it has been reported that clusterin is expressed at a low basal level in rat hepatocytes as determined by in situ hybridization and it is upregulated during hepatic growth and regression on hepatomitogen induction.[17,18] In previous studies, clusterin overexpression has been reported in HCC.[18] However, the significance of clusterin expression in nontumor sections of liver tissues has never been investigated.

Several studies have reported cytoplasmic overexpression of clusterin in human neoplasm using immunohistochemical staining. Kang et al[17] have shown clusterin expression in nonneoplastic liver tissues. Similarly, Scaltriti et al[14] have reported clusterin expression in the stromal components of nonneoplastic tissue as well as carcinoma of the prostate. Consistent with previous studies, our results showed similar clusterin expression in nonneoplastic liver tissue, and cytoplasmic with additional canalicular-secreted staining pattern in tumor cells of HCC tissues.

In the present study, 54% HCC tissues were positive for clusterin, while 38% nontumor adjacent liver tissues showed clusterin expression. Thus, the correlation between clusterin

Table 2

Univariate survival analysis of disease-free survival by the Kaplan-Meier method

Variables	3 y (%)	5 y (%)	10 y (%)	*p*
Age, y				
≤65 (n = 73)	33.3	24.8	18.7	0.309
>65 (n = 67)	31.3	20.4	12.8	
Sex				
Male (n = 113)	33.1	23.0	16.0	0.982
Female (n = 27)	29.6	20.7	13.8	
HBsAg				
(–) (n = 67)	37.3	24.0	17.9	0.221
(+) (n = 73)	27.7	21.1	13.0	
Anti-HCV				
(–) (n = 106)	31.4	22.6	17.2	0.897
(+) (n = 34)	35.3	22.1	14.7	
AFP level, ng/mL				
≤20 (n = 58)	39.6	24.5	19.6	0.162
>20 (n = 82)	28.0	21.3	13.3	
Albumin, g/dL				
≤4.0 (n = 80)	22.5	11.8	8.1	0.005[a]
>4.0 (n = 60)	45.0	37.0	25.9	
ALT, U/dL				
≤45 (n = 80)	35.4	22.9	16.9	0.439
>45 (n = 60)	28.3	21.5	13.7	
ICG				
(≤10%) (n = 58)	38.6	34.3	24.3	0.058
(>10%) (n = 82)	28.0	15.2	10.1	
Tumor size, cm				
≤5 (n = 80)	36.2	24.7	15.7	0.063
>5 (n = 60)	27.3	19.8	12.9	
Tumor number				
Single (n = 88)	39.2	25.8	17.3	0.02[a]
Multiple (n = 52)	20.8	16.7	12.5	
Macrovascular invasion				
(–) (n = 111)	36.9	24.4	16.7	<0.001[a]
(+) (n = 29)	15.0	15.0	11.3	
Microvascular invasion				
(–) (n = 52)	44.2	32.7	13.4	0.003[a]
(+) (n = 88)	25.3	16.9	14.4	
TNM stage				
I (n = 48)	43.7	31.0	12.2	0.005[a]
II (n = 47)	33.9	20.3	15.5	
III (n = 45)	18.5	16.2	13.9	
Liver cirrhosis				
(–) (n = 82)	34.6	30.3	23.2	0.256
(+) (n = 58)	29.3	13.3	6.5	
Surgical margin, cm				
≤1 (n = 97)	27.7	19.5	13.6	0.116
>1 (n = 43)	43.0	29.5	19.9	
Serum clusterin level, μg/mL				
(≤130.0) (n = 64)	38.2	23.6	18.6	0.324
(>130.0) (n = 76)	27.6	21.0	12.9	
Tumor clusterin				
(–) (n = 64)	31.1	23.9	17.7	0.767
(+) (n = 76)	33.5	21.4	13.3	
Nontumor clusterin				
(–) (n = 87)	37.9	30.1	22.1	0.001[a]
(+) (n = 53)	23.3	9.9	5.0	

AFP = α-fetoprotein level; ALT = alanine aminotransferase (normal range 0-40 U/L); anti-HCV = anti-hepatitis C virus antibody; HBsAg = hepatitis B surface antigen; ICG-15 = indocyanine green dye retention at 15 minutes after the intravenous injection (normal range 0-10%); TNM = tumor-node-metastasis.

[a]Statistically significance.

Table 3

Results of multivariate analysis of factors related to disease-free survival

Variables	β	SE	*p*	Exp(β) (95% CI)
Macrovascular invasion (+)	–0.774	0.252	0.002[a]	0.461 (0.281-0.756)
Microvascular invasion (+)	–0.479	0.215	0.026[a]	0.620 (0.407-0.944)
Nontumor clusterin (+)	–0.746	0.198	<0.001[a]	0.474 (0.322-0.698)

[a]Statistically significance.

expression pattern in nontumor tissue and worse prognosis, which was investigated in this study, has never been reported yet. The relationship assessed in our study can potentially be a secondary mechanism to liver carcinogenesis. We performed statistical comparison of clusterin overexpression in nontumor tissue with various clinicopathological parameters of patients with HCC. Accordingly, no significant difference was found in the clusterin expression pattern based on tumor size, number of tumor nodules, and occurrence of vascular invasion. Moreover, we did not observe any correlation between clusterin expression in nontumor liver tissue and the degree of deterioration of functional liver. Thus, the data suggested that increased clusterin expression can be related to the process of carcinogenesis rather than cirrhosis or fibrosis. Consequently, our results revealed that clusterin may play a significant role in the tumorigenesis of human liver. However, further studies are required to determine the underlying mechanisms in the functional role of clusterin in HCC.

Our study had some potential limitations. At first, it was a single institutional, retrospective study. Additionally, the sample size was relatively small. Third, our database was mainly based on the cohort of patients with HCC and predominant hepatitis B virus infection in Taiwan. Therefore, a study concerning the population with HCC and predominant HCV infection or a history of alcohol abuse should be considered. Thus, a large-scale prospective study is required to validate the results in a large population.

In conclusion, clusterin expression in nontumor liver tissue is an independent adverse prognostic factor related to postoperative disease-free survival in patients with HCC. Our data suggest that upregulated clusterin expression in nontumor liver tissue plays a significant role in tumorigenesis and thus can be utilized as a therapeutic target in postresectional HCC treatment.

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