Cancer Science
Japanese Cancer Association JCA
Open Access

CD44 variant 9 is a potential biomarker of tumor initiating cells predicting survival outcome in hepatitis C virus-positive patients with resected hepatocellular carcinoma

Anna Kakehashi,[1] Naomi Ishii,[1] Eiji Sugihara,[2] Min Gi,[1] Hideyuki Saya[2] and Hideki Wanibuchi[1]

[1]Department of Molecular Pathology, Osaka City University Graduate School of Medicine, Osaka; [2]Division of Gene Regulation, Institute for Advanced Medical Research, School of Medicine, Keio University, Tokyo, Japan

Key words

Biomarker, CD44v9, hepatocellular carcinoma, oxidative stress, TISC

Correspondence

Hideki Wanibuchi, Department of Molecular Pathology, Osaka City University Graduate School of Medicine, Asahi-machi 1-4-3, Abeno-ku, Osaka 545-8585, Japan. Tel: +81-6-6645-3735; Fax: +81-6-6646-3093; E-mail: wani@med.osaka-cu.ac.jp

Funding Information

Ministry of Health, Labor and Welfare of Japan; Japan Science and Technology Corporation.

Received October 23, 2015; Revised February 4, 2016; Accepted February 11, 2016

Cancer Sci **107** (2016) 609–618

doi: 10.1111/cas.12908

This study investigated whether the expression of CD44 variant 9 (CD44v9) might be a functional marker of tumor-initiating stem-like cells in primary hepatocellular carcinomas (HCCs) of hepatitis C virus (HCV)$^+$ patients and provide an indicator of patient survival, as well as associated mechanisms. A total of 90 HCV$^+$ HCC patients who underwent surgery from 2006 to 2011 were enrolled and monitored for 2–8 years. Expression of CD44v9 was validated immunohistochemically in all HCCs, followed by comparative proteome, survival, and clinicopathological analyses. CD44 variant 8–-10 was further evaluated in diethylnitrosamine-induced HCCs of C57Bl/6J mice. Focally localized CD44v$^+$ cells with a membranous staining pattern were detected in human HCV$^+$ and mouse HCCs. CD44v9$^+$ cells of HCCs were predominantly negative for Ki67 and P-p38, indicating decrease of cell proliferation in the CD44v9$^+$ tumor cell population, likely to be related to suppression of intracellular oxidative stress due to activation of Nrf2-mediated signaling, DNA repair, and inhibition of xenobiotic metabolism. CD44v9 IHC evaluation in 90 HCV$^+$ HCC cases revealed that positive expression was significantly associated with poor overall and recurrence-free survival, a younger age, poor histological differentiation of HCCs, and high alkaline phosphatase levels compared with patients with negative expression. CD44v9 is concluded to be a potential biomarker of tumor-initiating stem-like cells and a prognostic marker in HCV$^+$ HCC patients associated with Nrf2-mediated resistance to oxidative stress.

Hepatocellular carcinoma is the most common primary malignant tumors in liver, accounting for approximately 80% of all hepatic cancer cases worldwide. Surgical resection and liver transplantation are generally applied for early-stage HCCs, but the prognosis of HCC patients is generally poor due to high levels of tumor invasion and resistance to chemotherapy.[1] Accumulating evidence suggests that subpopulations of cancer cells with stem-like cell properties, so-called CSCs or TISCs, play a critical role in HCC development and progression.[2] Recent studies reported that dedifferentiated hepatocytes, hepatic oval cells, and bone marrow cells are the three major types of liver stem cells and CD133, CD44, CD90, OV6, epithelial cell adhesion molecule, and CD13 have been identified as specific antigenic markers for CSCs isolated from different human HCC cell lines or specimens.[2–4] Wnt, Hedgehog, Notch, TGF-β, and angiogenic signaling are main pathways that regulate HCC stem cell self-renewal, differentiation, and survival, and may be potential targets for novel therapeutic strategies of HCC.[2,4]

CD44 is a single-pass type I transmembrane protein that serves as a cell surface receptor and functions as a cellular adhesion molecule for hyaluronic acid, a major component of the ECM.[5] It is involved in a wide variety of physiological processes, such as leukocyte homing and activation, wound healing, and cell migration.[1,5] It has been shown that the existence of alternative splicing of CD44 variant exons generates many CD44 variant isoforms (CD44v), which function differentially.[5,6] Furthermore, CSCs with an epithelial phenotype predominantly express isoforms containing variant exons, whereas CSCs that have undergone an epithelial–mesenchymal transition downregulate these variant isoforms and upregulate expression of the standard CD44 isoform that contains no variant exons.[7] Expression of CD44v in some tissues appears to relate to tumor progression, and in particular to metastatic potential of some cancers.[1,8–10]

Expression of the CD44v6 isoform has been found to be significantly correlated with a poor prognosis and more aggressive stages of disease in patients with high-grade non-Hodgkin's lymphoma,[11] as well as stomach, colorectal, breast, and cervical cancers, with suggested potential as a CSC marker.[12–14] However, significant correlations between CD44v6 expression and clinicopathological factors in patients appear to be lacking. Recently, it was reported that interaction of CD44v8-10 with xCT (SLC7A11), a subunit of the

glutamate–cystine transporter system xc(−), stabilizes the latter and thereby potentiates the ability of cancer cells to promote glutathione synthesis and control the cell redox potential, making TISCs resistant to induction of ROS, this being important for CSC survival.[15,16] CD44 variant 9 has been reported to feature an xCT interaction site.[17] Furthermore, it was recently proposed as a potential predictive marker for recurrence in early gastric cancers.[18] Overexpression was found in metaplastic cells in gastric cancer under chronic inflammation conditions, suggesting induction in response to inflammation and possible promotion of metaplasia of the gastric epithelium.[19] To establish whether CD44v9 might be a novel reliable TISC marker for human HCC, in the present study we assessed its expression in HCV^+ human HCCs and DEN-induced mouse hepatomas. Proteome analysis of $CD44v9^+$ and $CD44v9^-$ HCCs and adjacent liver tissues was used for the investigation of CD44v9-related proteins, upstream regulators, molecular functions, and signaling and canonical pathways. Furthermore, the possible clinical significance of CD44v9 was investigated in 90 HCV^+ HCC patients by IHC analysis.

Materials and Methods

Chemicals. All reagents were obtained from Sigma (St. Louis, MO, USA) or Wako Pure Chemicals Industries (Osaka, Japan). N-nitrosodiethylamine was purchased from Sakai Research Laboratory (Fukui, Japan).

Institutional review board approval and informed consent. The present study was approved by the Osaka City University Graduate School of Medicine ethics committee (Osaka, Japan). Written informed consent was obtained from the patients prior to the study. The study was carried out in accordance with the principles of the Declaration of Helsinki.

Human tissue specimens, patients, and treatment. This prospective study included tissue specimens obtained from 90 HCV^+ patients with primary HCC undergoing surgery at Osaka City University Hospital (Osaka, Japan) from January 2006 to December 2011. All of these patients were histologically proven to have HCC and were enrolled in this study to assess the clinical significance of expression levels of CD44v9 in resected liver specimens of primary HCCs. Information on the features of the patients such as gender, age, body mass index, smoking history, tumor volume, and tumor differentiation were obtained from medical records (Table S1). There were 62 male and 28 female patients, with a median age of 72 years (range, 45–84 years) at the time of surgery. Histopathological analysis and staging were carried out in accordance with the Japanese classification of hepatocellular carcinoma.[20] The pathological diagnoses were made by at least two pathologists from the pathology department in our hospital according to the criteria of the general guidelines for primary liver cancer of the American Joint Committee on Cancer/International Union Against Cancer Staging Systems[21] and the Liver Cancer Study Group of Japan.[20,22] Fifty-four of the 90 patients were diagnosed with recurrence.

Animals, experimental design, and histopathology. A total of 32 male 7-day-old C57BL/6J mice (CLEA, Tokyo, Japan) were quarantined for 1 week before the start of the experiment. They were housed in an animal facility maintained on a 12:12 h (07:00–19:00) light : dark cycle, at a constant temperature of 23 ± 1°C and relative humidity of 44 ± 5%, and were given free access to tap water and food (Oriental MF pellet diet; Oriental Yeast Co., Tokyo, Japan). All experimental procedures were carried out following approval of the Animal Care and Use Committee of the Osaka City University Graduate School of Medicine.

A total of 32 male 14-day-old C57Bl/6J mice were divided into two experimental groups. Twenty-four animals underwent an i.p. injection of DEN (10 mg/kg b.w.) and eight mice (control) were injected with saline. At 38 weeks the mice were killed. Livers were macroscopically examined and separate portions fixed in 10% phosphate-buffered formalin for histopathological assessment and IHC analyses.

Immunohistochemistry and scoring. Single IHC for CD44v and double IHC for Ki67, P-p38, Nrf2, p62-SQSTM1, Keap1, and CD44v were carried out using the ABC method as described previously.[23] In double IHC, CD44v was visualized with 3,3′-diaminobenzidine tetrahydrochloride solution (DAKO, Tokyo, Japan) or alkaline phosphatase (Vectastain ABC-AP kit, Vector red; Vector Laboratories, Burlingame, CA, USA), and Ki-67, P-p38, Nrf2, p62-SQSTM1, and Keap1 with alkaline phosphatase (Vectastain ABC-AP kit, Vector blue; Vector Laboratories, Burlingame, CA, USA). CD44v was detected with rat mAbs against human CD44v9 (RV3; 1:500) and mouse CD44v8-10 (CD44v10-e16 [RM1]; 1:300), kindly provided by Prof. H. Saya (Keio University, Tokyo, Japan), generated as previously described.[17] Ki67 was detected with rabbit mAbs (1:300; Abcam, Tokyo, Japan) and P-p38 with mouse mAbs (1:150; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Rabbit polyclonal antibodies for Nrf2 (1:100; Abcam, Tokyo, Japan), p62-SQSTM1 (1:1000; MBL Co., Osaka, Japan), and Keap1 (1:200; Abcam, Tokyo, Japan) were applied. The membranous CD44v expression levels in cancer cells of the tumors were interpreted using guidelines published in previous studies.[24] For human CD44v9, the results were graded from 0 to 3+ as follows: 0, no staining; 1+, 1–25% staining; 2+, 26–50% staining; 3+, >50% of the specimen was stained. Ki67 and P-p38 IHC scores were calculated in $CD44v9^+$ and $CD44v9^-$ tumor areas as the averages of the percentage of $Ki67^+$ or $P\text{-}p38^+$ cells in each of three high-power fields of areas containing tumor cells.

QSTAR Elite LC-MS/MS. Of the 90 HCV^+ HCC patients, five cases each of $CD44v9^+$ and $CD44v9^-$ HCCs were randomly selected for proteome analysis. Samples from dissected 10% phosphate-buffered formalin-fixed HCCs and adjacent normal-appearing tissues were prepared in liquid tissue buffer (AMR, Tokyo, Japan), treated with heat at 90°C for 90 min and digested with trypsin at 37°C for 16–18 h. Proteome analysis was carried out with the selected $CD44v9^+$ and $CD44v9^-$ HCCs and adjacent normal tissue samples (20 μg each) with a DiNa-AI nano LC System (KYA Technologies, Tokyo, Japan) coupled to a QSTAR Elite hybrid mass spectrometer (AB Sciex, Concord, ON, Canada) through a NanoSpray ion source (AB Sciex), as previously described.[25] For the quantitative MS/MS analysis, labeling was carried out with 4-plex iTRAQ reagents.[25] The LC-MS/MS and ProteinPilot analyses results of five $CD44v9^+$ and five $CD44v9^-$ patients were averaged and further underwent IPA (Ingenuity Systems, Mountain View, CA, USA) to investigate protein molecular functions, localization, and identification of networks of interacting proteins, as well as functional groups and pathways and altered upstream regulators.

Statistical analyses. All statistical analyses were carried out using SPSS statistics version 19.0 (SPSS, Chicago, IL, USA). Statistical significance of any associations between CD44v9 immunohistochemical staining and various clinicopathological variables was evaluated using χ^2- and Fisher's tests. Survival curves were calculated from the day of surgery to relapse,

death, or the last follow-up observation using the Kaplan–Meier method, and differences in overall and recurrence-free survival curves were assessed with the log–rank test. In all applied analyses, *P*-values < 0.05 were considered statistically significant. Univariate and multivariate Cox proportional hazard model analyses were carried out to calculate HRs and to determine associations between clinicopathologic variables and disease-specific mortality. Univariate analysis describes survival with respect to the factor under investigation, but necessarily ignores the impact of any others. We therefore undertook a multivariate survival analysis to compare prognostic factors of survival after adjustment for the impact of other factors. Different potential predictive variables were evaluated in the univariate analysis: CD44v9 (positive *vs* negative), age (>70 *vs* ≤70 years), diabetes (positive *vs* negative), fibrosis (stages 3 and 4 *vs* stages 1 and 2), aspartate transaminase value (>34 *vs* 13–33 IU/L), alanine aminotransferase value (>28 *vs* 6–27 IU/L), ALP value (>360 *vs* 115–359 IU/L), tumor size (≥2 *vs* <2 cm^3), vessel invasion (positive *vs* negative), infiltration to capsule by tumor cells (positive *vs* negative), tumor differentiation (poor *vs* well and moderate), T category (T2–4 *vs* T1), stage (stages 2–4 *vs* stage 1), and recurrence (positive *vs* negative). A Cox proportional hazard regression model was used to estimate prognostic factors associated with survival in the multivariate analysis.

Results

Representative results of CD44v9 IHC in HCV^+ human and mouse HCCs. Representative CD44v9 immunohistochemical staining is illustrated in Fig. 1(A). In the specimens of 90 patients examined, CD44v9 was strongly elevated in 24 cases (26.7%; score 3+), moderately expressed in 16 cases (17.8%; score 2+), weakly expressed in 25 cases (27.8%: score 1+), and negative in 25 cases (27.7%; score 0). Although CD44v9 was not detectable in HCC patients of the $CD44v9^-$ group and non-tumor areas of all patients, it was overexpressed in several well, moderately, and most strongly, poorly differentiated HCCs with focal heterogeneous expression patterns ($CD44v9^+$ group). In poorly differentiated tumors we observed single giant multinuclear cells with eosinophilic cytoplasm, which were positive for CD44v9. Significant correlations were identified between CD44v9 positivity with younger patient age and poor tumor differentiation (Table 1). In the livers of colorectal cancer patients, used as positive control for CD44v9, 100% of metastatic lesions were stained, while in the normal-appearing liver area, no $CD44v9^+$ cells were observed.

Histopathological analysis and assessment of CD44v positivity in C57Bl/6J mice. During this study, no animals died and no significant changes in body weight, food, or water intake were observed. The number of putative preneoplastic foci observed in C57Bl/6J mice treated with DEN was 1.24 ± 0.90 no/cm^2. The incidence of HCA and HCC of mice observed at week 38 after DEN initiation were 50% (12 mice) and 12.5% (3 mice), respectively, and multiplicities were 1.13 ± 1.36 and 0.13 ± 0.35 no./mouse. A mixed-type tumor, hepatocholangiocarcinoma (1 mouse, 4%) was found in the DEN group. Representative CD44v IHC staining is illustrated in Fig. 1(B). Assessment of CD44v showed that HCCs but not HCAs or putative preneoplastic foci of mice contained focal regions or single cells positive for CD44v (Fig. 1Ba,b,e–h). No CD44v overexpression was obvious in adjacent non-tumor areas, and normal biliary duct-

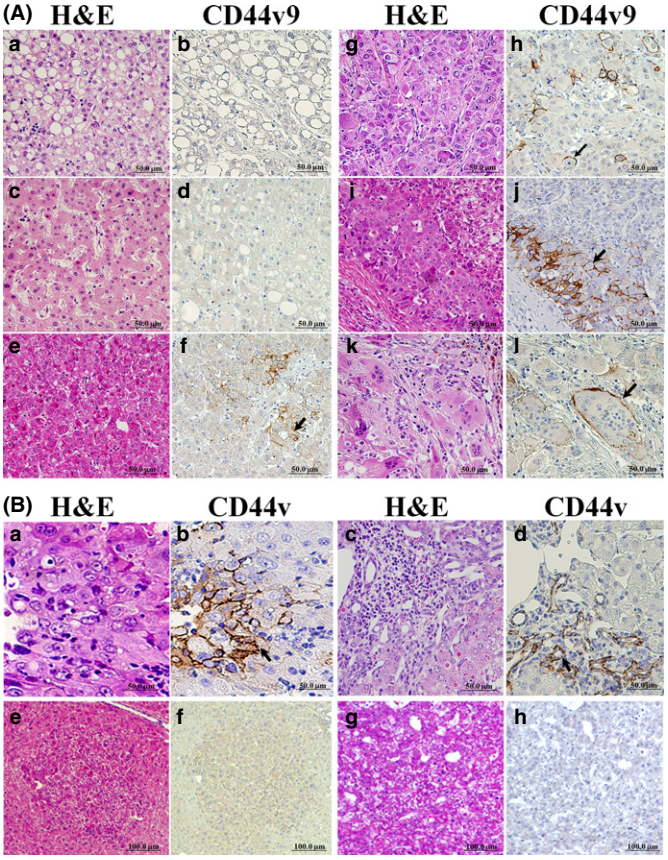

Fig. 1. Immunohistochemistry for CD44 variant 9 (CD44v9) in hepatitis C virus-positive human (A) and mouse (B) hepatocellular carcinomas (HCCs). (A) $CD44v9^-$ non-recurrent HCC (a,b), normal-appearing liver (c,d), and well (e,f), moderately (g,h), and poorly (i,j and k,l) differentiated $CD44v9^+$ recurrent HCCs. Staining in human HCC used H&E (a,c,e,g,i,k) and CD44v9 (b,d,f,h,j,l). Note $CD44v9^+$ cells (arrows) and positive multinuclear cells (l) in HCCs. (B) In mouse HCCs, note $CD44v^+$ cells (a,b) and positive proliferative ductular epithelial cells in the mixed type tumor (c,d) (arrows). $CD44v^-$ basophilic foci (e,f) and hepatocellular adenoma (HCA) (g,h) in mouse liver (serial sections).

tular cells were negative for CD44v. Almost all proliferating ductular epithelial cells (>90%) within the mixed-type tumor were positive for CD44v (Fig. 1Bc,d). In this experiment, no bile duct proliferative lesions in the normal-appearing liver tissue were found.

Observed correlations between Ki67, P-p38, Nrf2, p62-SQSTM1, Keap1, and CD44v in human and mouse HCCs. Next, we examined the expression of Ki67, Nrf2, p62-SQSTM1, Keap1, and activation of $p38^{MAPK}$, a major target of ROS, in cancer tissues to assess the role of CD44v in the regulation of intracellular oxidative stress. The representative pictures of double immunostaining for target proteins and CD44v are presented in Fig. 2. Ki67 immunopositive nuclei were barely detectable in $CD44v9^+$ regions of human HCCs ($3.41 \pm 2.06\%$, $P < 0.05$), $CD44v9^+$ colorectal cancer metastases to the liver, and mouse $CD44v^+$ HCC regions ($4.24 \pm 7.35\%$, $P < 0.05$), but were apparent in human ($17.63 \pm 9.48\%$) and mouse ($30.00 \pm 12.00\%$ $CD44v^-$ HCC areas (Fig. 2Aa–d,Bb,Ca,Db). Similarly, P-p38 staining was not obvious in human ($1.80 \pm 2.65\%$, $P < 0.05$) or mouse ($1.52 \pm 2.62\%$, $P < 0.05$) $CD44v^+$ HCC regions but was detected in human ($45.92 \pm 38.77\%$) and mouse ($24.00 \pm 12.12\%$) $CD44v^-$ HCC areas (Fig. 2Ae,Bc,Cc,Dc). Furthermore, it was found

Table 1. Correlation between CD44 variant 9 expression and clinicopathological variables

Factors	No. of patients (%)			
	Total	Positive (n = 64)	Negative (n = 26)	P-value†
Age				
>70	68	44 (65)	24 (35)	0.018
≤70	22	20 (91)	2 (9)	
Gender				
Male	62	44 (71)	18 (29)	0.964
Female	28	20 (71)	8 (29)	
Smoking				
Smoker	50	33 (66)	16 (64)	0.232
Non-smoker	40	31 (77)	9 (23)	
Drinking				
Drinker	36	25 (69)	11 (31)	0.776
Non-drinker	54	39 (72)	15 (28)	
Diabetes				
Positive	20	17 (85)	3 (15)	0.120
Negative	70	47 (67)	23 (33)	
Tumor size, cm^3				
≤2	55	39 (71)	16 (29)	0.871
>2	35	25 (71)	10 (29)	
T category (pT)				
T1	25	16 (64)	9 (36)	0.356
T2–T4	65	48 (74)	17 (26)	
Im				
Positive	14	10 (71)	4 (29)	0.977
Negative	76	54 (71)	22 (29)	
pM				
Positive	1	0 (0)	1 (100)	0.115
Negative	89	64 (72)	25 (28)	
pB				
Positive	1	1 (100)	0 (0)	0.522
Negative	89	63 (71)	26 (29)	
Venous invasion				
Positive	25	20 (80)	5 (20)	0.187
Negative	65	44 (68)	21 (32)	
Fc inf				
Positive	54	41 (76)	13 (24)	0.159
Negative	36	23 (64)	13 (36)	
Differentiation‡				
Well	8	5 (62)	3 (38)	0.033
Moderate	35	21 (60)	14 (40)	
Poor	47	38 (81)	9 (19)	
Clinical stage§				
I	25	16 (64)	9 (36)	0.251
II	41	30 (73)	11 (27)	
III	21	16 (76)	5 (24)	
IV	3	2 (67)	1 (33)	
Recurrence				
Positive	54	41 (76)	13 (24)	0.159
Negative	36	23 (64)	13 (44)	
Cirrhosis				
Stages 1 and 2	41	28 (68)	13 (32)	0.589
Stages 3 and 4	49	36 (73)	13 (27)	
AST, IU/L				
13–33	23	16 (70)	7 (30)	0.850
>34	67	48 (72)	19 (28)	
ALT, IU/L				
6–27	31	21 (68)	10 (32)	0.609
>28	59	43 (73)	16 (27)	
ALP, IU/L				
115–359	60	39 (65)	21 (35)	0.049¶
>360	30	25 (83)	5 (17)	

†Pearson's χ2-test. ‡Well and moderately differentiated versus poorly differentiated. §Stage I versus stages II–IV. No lymph node metastases were detected in any patient. ¶Fisher's test. ALP, alkaline phosphatase; ALT, alanine transferase; AST, aspartate transferase; Fc inf, infiltration to capsule; im, intrahepatic metastasis; pM factor, pathological M factor; pT factor, pathological T factor, pB factor, pathological B factor (bile duct invasion).

that CD44v positivity was correlated with Nrf2, p62-SQSTM1, and Keap1 overexpression in both human and mouse HCCs (Fig. 2Bd–f,Dd–f).

Association of CD44v9 staining with clinicopathological variables. To evaluate the correlation between CD44v9 expression and clinicopathological outcome, its expression was assessed by IHC in 90 HCV^+ HCC patients. The relationship between CD44v9 overexpression and various clinicopathological parameters including pathological stage, TNM factors, and tissue differentiation, venous and bile duct invasion of human HCCs was analyzed using the χ^2-test (Table 1). No statistically significant associations between increased CD44v9 expression (scores 0, 1+ to 3+) with gender, smoking, drinking, past history of diabetes, stage of cirrhosis, tumor size, the established prognostic factor of pT, pB, and pM status, or clinicopathological stage were found (Table 1). No lymph node metastases (pN status) were observed in any HCV^+ HCC patients. Interestingly, positivity and negativity of membranous CD44v9 expression were significantly associated with younger age (≤70 *vs* >70 years; $P = 0.018$) and poor histological tumor differentiation (poorly *vs* well and moderately; $P = 0.033$) (Fig. S1). Furthermore, a positive correlation was found between ALP elevation in the blood and CD44v9 expression (>360 *vs* 115–359 IU/L, $P = 0.049$) (Table 1, Fig. S1). Moreover, positive CD44v9 expression in tumors tended to be associated with venous invasion (positive *vs* negative, $P = 0.187$), infiltration of the capsule (positive *vs* negative, $P = 0.159$), and recurrence (positive *vs* negative, $P = 0.159$) (Table 1).

Prognosis. Next, univariate survival analysis with recurrence-specific survival curves was carried out, according to the Kaplan–Meier method, and differences in overall and recurrence-free survival were assessed with the log–rank test (Fig. 3). Importantly, positive CD44v9 expression was associated with poorer overall ($P = 0.022$, log–rank test) and recurrence-free ($P = 0.049$, log–rank test) survival compared with negative expression (Fig. 3A,B). Moreover, median recurrence-free survival periods for patients with positive and negative CD44v9 expression were 1016 days and 1370 days, respectively. The overall 100-month (3000-day) survival rates for patients with positive and negative CD44v9 expression were 54% and 84%, respectively. Moreover, median recurrence-free survival for patients with positive and negative

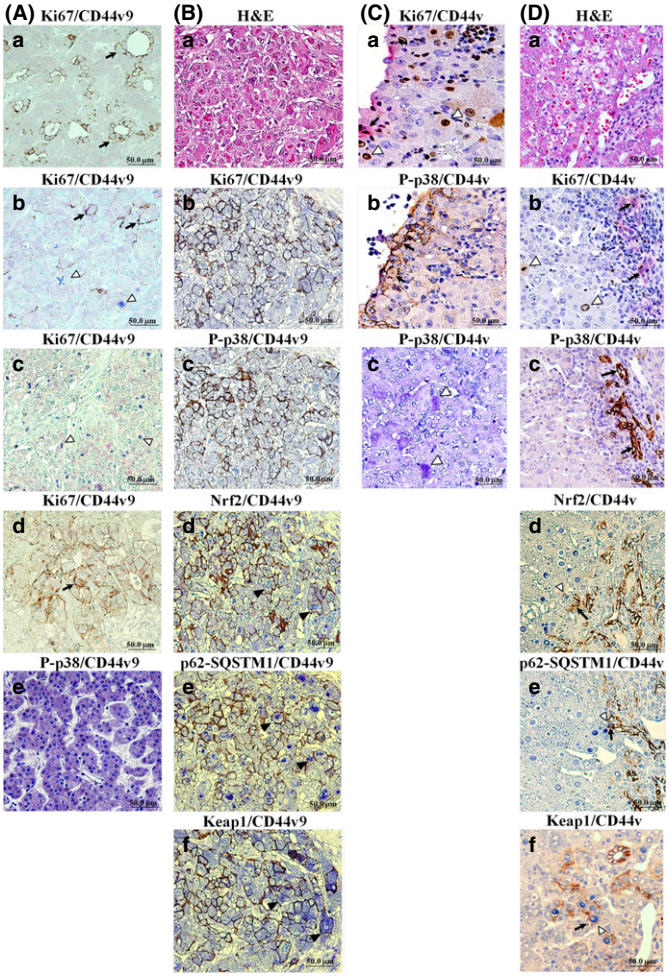

Fig. 2. Double immunohistochemistry (IHC) for CD44v (brown/black) and Ki67, phospho-p38 (P-p38), nuclear factor (erythroid-derived 2)-like 2 (Nrf2), p62-sequestosome 1 (SQSTM1), or Kelch-like ECH-associated protein 1 (Keap1) (blue) in representative cases of recurrent HCV$^+$ human and mouse hepatocellular carcinomas (HCCs). (A) IHC for human CD44 variant 9 (CD44v9; black arrows) and Ki67 or P-p38 (white arrowheads). (a) CD44v9$^+$ and Ki67$^-$ moderately differentiated HCC. (b) Independent CD44v9$^+$ and Ki67$^+$ cells in moderately differentiated HCC. (c) CD44v9$^-$ and Ki67$^+$ moderately differentiated HCC. (d) CD44v9$^+$ and Ki67$^-$ colorectal adenocarcinoma metastatic lesion in the liver (positive control). (e) CD44v9$^-$ and P-p38$^+$ human HCC. (B) H&E staining (a) and double IHC in serial sections for Ki67 (b), P-p38 (c), Nrf2 (d), p62-SQSTM1 (e), or Keap1 (f) and CD44v9 in human poorly differentiated HCC. (C) Double IHC for Ki67 (a,b), P-p38 (c), and CD44v in mouse HCC. (D) H&E staining (a) and double IHC for Ki67 (b), P-p38 (c), Nrf2 (d), p62-SQSTM1 (e), Keap1 (f), and CD44v in mouse mixed-type tumor. Note the inverse correlation between CD44v and Ki67 or P-p38 and the positive correlations between Nrf2, p62-SQSTM1, Keap1, and CD44v9 expression (black arrowheads).

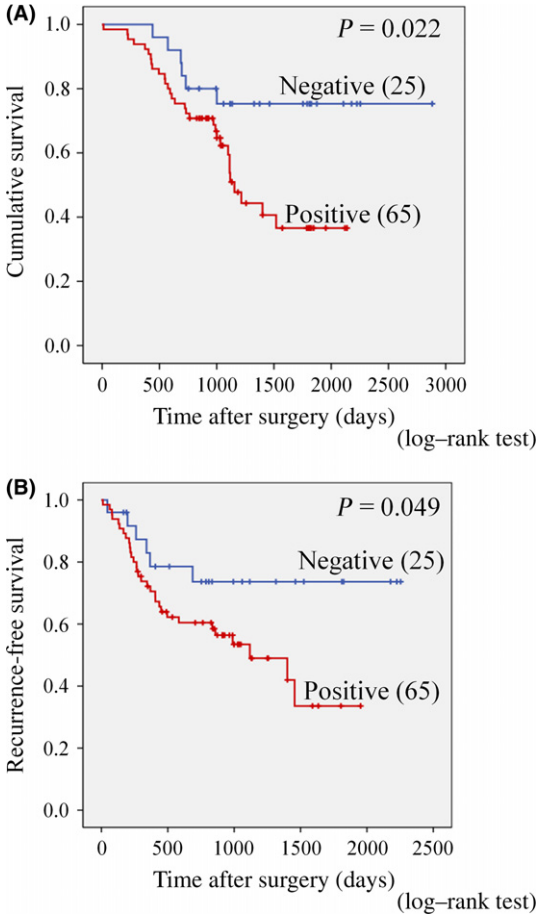

Fig. 3. Kaplan–Meier curves evaluating differences in overall (A) and recurrence-free (B) survival in hepatitis C virus-positive patients with hepatocellular carcinoma depending on CD44 variant 9 expression. Significant differences in overall and recurrence-free survival were observed for patients with CD44 variant 9 positive (n = 65) and negative (n = 25) expression (log–rank test).

CD44v9 expression was 736 days and 934 days, respectively. The recurrence-free 2500-day survival rates for patients with positive and negative CD44v9 expression were 38% and 44%, respectively. According to the results of univariate analysis, poor survival of patients was significantly associated with increased CD44v9 expression (HR, 2.781; 95% confidence interval, 1.155–6.694, P = 0.022), higher T category (T2–4 *vs* T1, P = 0.031), clinicopathological stage (II–IV *vs* I, P = 0.031), tumor size (>2 *vs* ≤2 cm^3, P = 0.009), venous invasion (positive *vs* negative, P < 0.0001), infiltration to capsule (positive *vs* negative, P = 0.047), poor tumor differentiation (poorly *vs* well and moderately, P = 0.037), and recurrence (positive *vs* negative, P = 0.015) (Table 2).

Multivariate analysis was performed using the Cox proportional hazards model for all significant variables in the univariate analysis. Survival of HCV$^+$ HCC patients was significantly associated with CD44v9 expression (HR, 2.536; 95% confidence interval (CI), 0.964–6.672, P = 0.049), venous invasion (positive *vs* negative, P = 0.026), infiltration of the capsule (positive *vs* negative, P = 0.047) and recurrence (positive *vs* negative, P = 0.009) (Table 2). Based on these results, CD44v9 expression was proven to be an independent prognostic factor for human HCV$^+$ HCC survival.

Comparison of HCC proteomes of CD44v9$^+$ and CD44v9$^-$ patients. The results of LC-MS/MS and IPA analyses of differentially expressed proteins in CD44v9$^+$ and CD44v9$^-$ HCCs as compared to adjacent liver tissues are presented in Table 3. In all CD44v9$^+$ HCCs, we observed that many genes related to xenobiotic metabolism including various cytochrome P450 isoenzymes and epoxide hydrolase 2 were downregulated, while genes involved in Nrf2-mediated signaling and degradation of superoxide radicals, such as NQO1 (8.22-fold upregulation) and SOD2 (5.02-fold upregulation) were strongly elevated. We also observed that β-catenin, DNA repair proteins APEX nuclease 1 and poly(ADP ribose) polymerase 1, vimentin, and pro-

Table 2. Univariate and multivariate analyses of survival in hepatitis C virus-positive patients with resected hepatocellular carcinoma (n = 90)

Clinicopathological features	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
CD44v9						
Positive versus negative	2.781	1.155–6.694	0.022	2.536	0.964–6.672	0.049
Age, years						
≤70 versus >70	1.650	0.688–3.955	0.2620		NA	
Tumor size, cm^3						
>2 versus ≤2	2.433	1.244–4.756	0.0090		NA	
T category						
T2–4 versus T1	2.831	1.102–7.270	0.0310		NA	
Intrahepatic metastasis						
Positive versus negative	2.237	1.082-4.627	0.0300		NA	
Venous invasion						
Positive versus negative	1.909	0.630–5.789	<0.0001	2.450	1.116–5.379	0.026
Infiltration to capsule						
Positive versus negative	2.055	1.011–4.176	0.0470	2.399	1.010–5.698	0.047
Differentiation						
Poor versus well/moderate	2.125	1.046–4.314	0.0370		NA	
Stage						
II–IV versus I	2.831	1.102–7.270	0.0310		NA	
Recurrence						
Positive versus negative	2.770	1.216–6.310	0.0150	3.383	1.357–8.434	0.009
Cirrhosis						
Stage 3/4 versus stage 1/2	1.286	0.516–3.206	0.3790		NA	
AST, IU/L						
>34 versus 13–33	0.988	0.478–2.041	0.9740		NA	
ALT, IU/L						
>28 versus 6–27	0.965	0.491–1.896	0.9170		NA	
ALP, IU/L						
>360 versus 115–359 IU/L	0.850	0.420–1.721	0.6520		NA	

Both multivariate and univariate analyses used Cox proportional-hazards regression. Variables were adopted in multivariate analysis for their prognostic significance by univariate analysis. ALP, alkaline phosphatase; ALT, alanine transferase; AST, aspartate transferase; CD44v9, CD44 variant 9; NA, not available. No lymph node metastases were detected in any patient.

teins involved in actin cytoskeleton organization and glucose metabolism were overexpressed, whereas keratins 8 and 18 were downregulated only in $CD44v9^+$ HCCs (Table 3).

Comparison analysis of protein functions by IPA indicated that in $CD44v9^+$ human HCCs, numerous proteins with altered expression were involved in activation of cellular invasion, migration, synthesis of nitric oxide, metabolism of nucleotides (z-score ≥2.0), and suppression of generation and quantity of ROS, lipid oxidation, fatty acid metabolism, DNA damage, and cell death (z-score ≤2.0) (Table S2). Furthermore, analysis of altered canonical pathways showed that most upregulated proteins were involved in Nrf2-mediated oxidative stress responses and superoxide radicals degradation, protein kinase A signaling, glycolysis, gluconeogenesis, tricarboxylic acid cycle, fatty acid α-oxidation, signaling by Rho family GTPases, hepatic fibrosis/hepatic stellate cell activation, gap junction signaling, DNA double-strand break repair by nonhomologous end joining, and DNA repair of oxidative modifications (Table S3). In contrast, xenobiotic metabolism and p53 signaling pathways were suppressed in $CD44v9^+$ HCCs.

Ingenuity pathway analysis of upstream regulators indicated that CD44v9 positivity resulted in significant activation of Nrf2, TGFB1, platelet-derived growth factor BB, epidermal growth factor receptor, basic fibroblast growth factor 2, vascular endothelial growth factor A, IL1A and IL1B, and low-density lipoprotein, and significant inhibition of hepatocyte nuclear factor 4A, transcription factor E2F1, WNT1-inducible-signaling pathway protein 2, and miR-1-3p miRNA (Fig. 4, Table S4). Furthermore, we found a tendency for inhibition of pregnane X receptor, constitutive androstane receptor (CAR), and CCAAT/enhancer binding protein (C/EBP).

Discussion

This study provided the first experimental evidence of focal overexpression of CD44v9 in human HCV^+ HCCs and CD44v8-10 in mouse HCCs with similar membranous staining patterns. The absence of $CD44v^+$ cells in mouse preneoplastic foci or HCAs indicated that formation of $CD44v^+$ TISCs is a late event in mouse hepatocarcinogenesis. Importantly, positive CD44v9 expression was associated with poorer overall and recurrence-free survival, younger age, and poor histological tumor differentiation, as compared with negative expression, indicating that CD44v9 could be a novel potential TISC biomarker and independent prognostic factor for human HCV^+ HCC. Furthermore, a positive correlation was found between ALP elevation in the blood and CD44v9 expression in HCCs, which could reflect increased invasion activity of $CD44v9^+$ cells and might have diagnostic significance.

It was recently reported that expression of CD44v9 in primary gastric cancer tissue could serve as an indicator of recurrence in early gastric cancers patients.[18] In addition, it has

Table 3. Differentially expressed proteins in CD44 variant 9 (CD44v9)$^{+}$ and CD44v9^{-} hepatocellular carcinomas, identified by liquid chromatography MS/MS

Protein name	Symbol	GI number/ Swiss-Prot accession	Ratio		Location	Function(s)
			CD44v9 (–)	CD44v9 (+)		
NAD(P)H dehydrogenase, quinone 1	NQO1	118 607	–	8.22	C	XM, NO S
Superoxide dismutase 2, mitochondrial	SOD2	134 665	–	5.02	C	RSR
Epoxide hydrolase 2, cytoplasmic	EPHX2	67 476 665	–	0.45	C	XM, ROS M
Flavin containing monooxygenase 3	FMO3	6 166 183	0.62	0.55	C	XM
Flavin containing monooxygenase 5	FMO5	1 346 021	–	0.70	C	XM
Cytochrome P450, fam. 2, subfam. C, polypep. 8	CYP2C8	117 225	–	0.28	C	XM, AAM, ORP
Cytochrome P450, fam. 2, subfam. C, polypep. 9	CYP2C9	6 686 268	0.60	0.27	C	XM, AAM, ORP
Cytochrome P450, fam. 2, subfam. D, polypep. 6	CYP2D6	84 028 191	–	0.50	C	XM, ORP
Cytochrome P450, fam. 2, subfam. E, polypep. 1	CYP2E1	117 250	–	0.66	C	XM, EM, ORP
Cytochrome P450, fam. 3, subfam. A, polypep. 4	CYP3A4	116 241 312	0.41	0.32	C	XM, ORP
Cytochrome P450, fam. 3, subfam. A, polypep. 5	CYP3A5	117 157	–	0.57	C	XM, ORP
Cytochrome P450, fam. 4, subfam. A, polypep. 11	CYP4A11	2 493 371	–	0.21	C	XM, AAM, ORP
Cytochrome P450, fam. 4, subfam. F, polypep. 3	CYP4F3	56 757 430	–	0.67	C	XM, AAM, ORP
Cytochrome P450, fam. 8, subfam. B, polypep. 1	CYP8B1	G3V8J2	–	0.57	C	XM, BAM, ORP
Carboxylesterase 1	CES1	119 576	–	0.45	C	XM
Catechol-O-methyltransferase	COMT	116 907	–	0.56	C	MET, XM, ESM
Glutathione S-transferase alpha 1	GSTA1	121 730	0.58	0.43	C	XM, GM
Thioredoxin	TXN	135 773	1.63	1.97	C	ORP, NO R
Catenin (cadherin-ass. protein), beta 1, 88 kDa	CTNNB1	461 854	–	1.65	N	TRA
Prothymosin, alpha	PTMA	135 834	–	1.70	N	TRA
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta	YWHAH	1 345 593	1.32	1.68	C	TRA, ST
Heat shock 70-kDa protein 5 (glucose-reg.prot.,78 kDa)	HSPA5	14 916 999	1.31	1.69	C	PF, A(–)
Heat shock 70-kDa protein 9 (mortalin)	HSPA9	21 264 428	1.29	2.27	C	PF, A(–)
Calnexin	CANX	543 920	1.88	1.68	C	PF
Transketolase	TKT	1 729 976	–	3.60	C	CM
Glucose-6-phosphate dehydrogenase	G6PD	116 242 483	–	3.23	C	CM, GLM, LM
Hexokinase domain containing 1	HKDC1	Q2 TB90	–	1.85	C	CM, GLM
Enolase 1, (alpha)	ENO1	119 339	–	1.64	C	CM, GLM, TRA
Carnitine O-acetyltransferase	CRAT	215 274 265	–	2.03	C	LM, CAM, T
UDP-glucose pyrophosphorylase 2	UGP2	59 803 098	0.52	0.43	C	CM, GLM, XM
Arginase 1	ARG1	12 230 985	0.51	0.38	C	RD, UC
Argininosuccinate synthase 1	ASS1	20 141 195	0.32	0.36	C	RD, UC
Carbamoyl-phosphate synthase 1, mitochondrial	CPS1	4033 707	0.29	0.33	C	RD, UC
Ornithine carbamoyltransferase	OTC	84 028 235	0.70	0.60	C	RD, UC
Vimentin	VIM	55 977 767	–	1.61	C	CO
Keratin 18	KRT18	125 083	–	0.62	C	CO
Keratin 8	KRT8	90 110 027	–	0.58	C	CO
Cofilin 1 (non-muscle)	CFL1	116 848	–	1.65	N	ACO
Solute carrier fam. 9, subfam. A, mem. 3 reg. 1	SLC9A3R1	41 688 557	–	1.62	PM	ACO, CP(–)
Transgelin 2	TAGLN2	586 000	1.25	2.18	C	ACO
Periostin, osteoblast specific factor	POSTN	93 138 709	–	3.52	ES	CA
Fibronectin 1	FN1	P04937	2.60	2.04	ES	CA, EMO
APEX nuclease (multifunct. DNA repair enzyme) 1	APEX1	113 984	–	1.67	N	DNAR, ReOS
Poly (ADP-ribose) polymerase 1	PARP1	130 781	–	1.60	N	DNAR, BER
Synaptophysin-like 1	SYPL1	48 474 786	–	2.90	PM	T

Table 3 (Continued)

Protein name	Symbol	GI number/ Swiss-Prot accession	Ratio		Location	Function(s)
			CD44v9 (−)	CD44v9 (+)		
GTPase activating protein binding protein 1	G3BP1	14 916 572	–	3.14	N	T, ST
RAB35, member RAS oncogene family	RAB35	Q15286	–	1.71	C	T, ST
Apolipoprotein E	APOE	114 039	6.36	1.54	ES	T, ST
Fatty acid binding protein 1, liver	FABP1	119 808	0.66	0.23	C	T ReOS
DEAD (Asp-Glu-Ala-Asp) box polypeptide 39A	DDX39A	61 212 932	1.83	1.76	N	mRNA SPT

A(−), negative regulation of apoptosis; AAM, arachidonic acid metabolism; ACO, actin cytoskeleton organization; BAM, bile acid metabolism; BER, base excision repair; C, cytoplasm; CA, cell adhesion; CAM, carnitine metabolism; CM, carbohydrate metabolism; CO, cytoskeleton organization; CP(−), negative regulation of cell proliferation; DNAR, DNA repair; E, enzyme; EM, ethanol metabolism; EMO, ECM organization; ES, extracellular space; ESM, estrogen metabolism; GM, glutathione metabolism; GLM, glucose metabolism; MET, methylation; mRNA SPT, mRNA splicing, processing, and transport; N, nucleus; NO R, response to nitric oxide; NO S, nitric oxide synthesis; ORP, oxidation-reduction process; PF, protein folding; PM, plasma membrane; RD, response to drug; ReOS, response to oxidative stress; ROS M, reactive oxygen species metabolism; RSR, removal of superoxide radicals; ST, signal transduction; T, transport; TRA, transcription; UC, urea cycle.

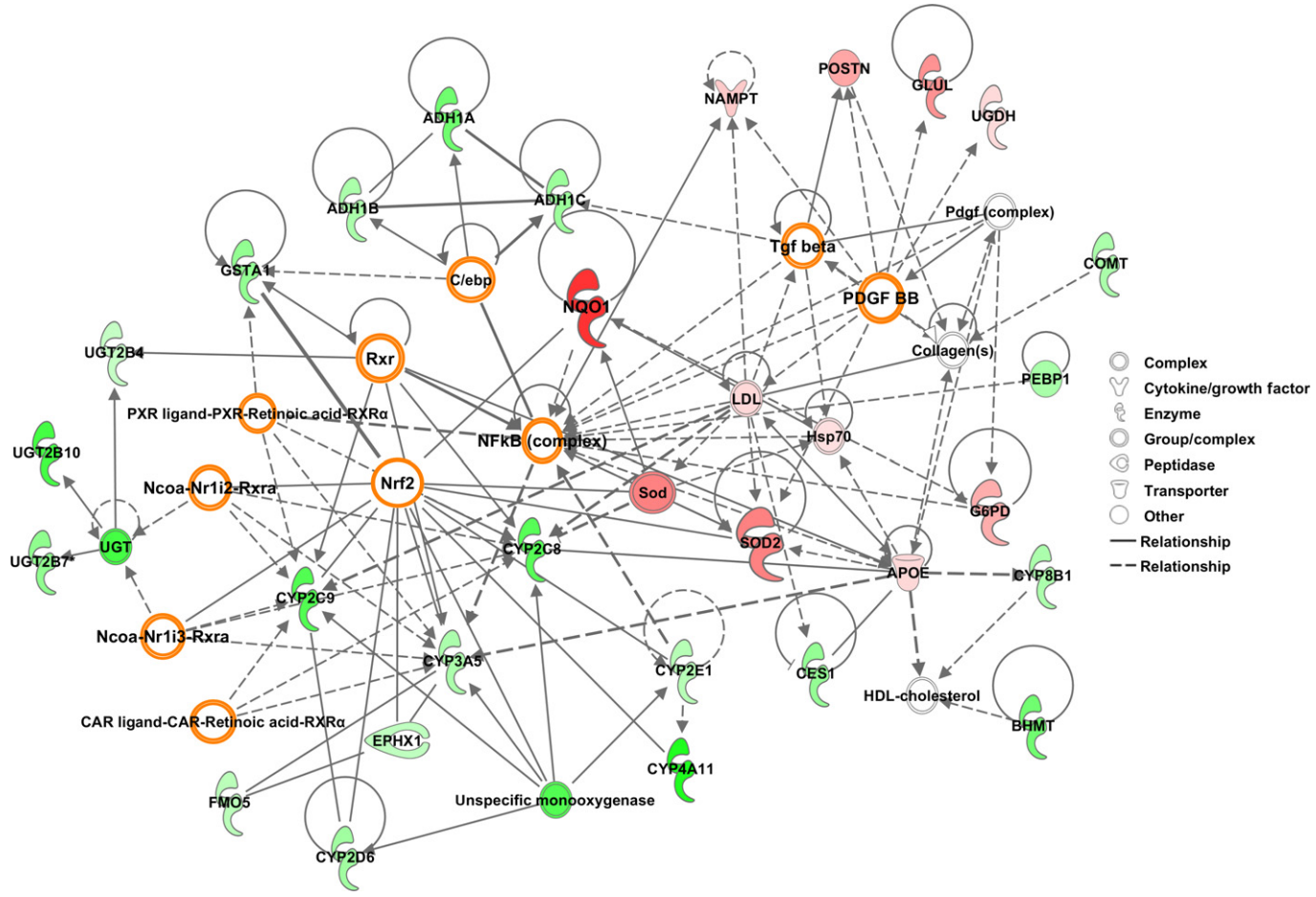

Fig. 4. Signaling pathways involving differentially expressed proteins in CD44 variant 9-positive hepatocellular carcinomas. Note activation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), with strong overexpression of its downstream proteins NAD(P)H quinone oxidoreductase (NQO1) and superoxide dismutase 2 (SOD2), as well as activation of transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) and inhibition of pregnane X receptor (PXR), constitutive androstane receptor (CAR), and CCAAT/enhancer binding protein (C/EBP). The illustration shows proteins with increased (red) and decreased (green) expression in CD44 variant 9-positive hepatocellular carcinomas.

been reported that, in lung cancer patients, CD44v8-10 mRNA and protein are predominantly expressed in non-small-cell lung carcinomas, while non-tumorous tissues principally express CD44s and small-cell lung carcinomas express either CD44s or no detectable CD44. Thus, CD44v8-10 was concluded to be the dominant splicing isoform in non-small-cell lung carcinomas and can be practically utilized for diagnosis and as a therapeutic target.[26]

Interestingly, Nrf2 downstream proteins such as NQO1 and SOD2, participating in degradation and removal of ROS, were greatly overexpressed in human CD44v9$^+$ HCCs, pointing to an important role for Nrf2 activation in TISCs. We further observed the concomitant overexpression of Nrf2, p62-SQSTM1, Keap1, and CD44v9, being an indicator of their interrelation and activation of Nrf2 by p62-SQSTM1. Nrf2 is a potent transcription activator that recognizes a unique DNA sequence known as the antioxidant response element, and *xCT* is one of its downstream genes.[27] Furthermore, CD44v9 positivity was associated with suppressed expression of numerous proteins involved in xenobiotic metabolism, which are controlled by hepatocyte nuclear factor 4A, E2F1, constitutive androstane receptor, and PXR upstream regulators.[28,29] These data are in line with previously published results demonstrating involvement of CD44v8-10 in potentiation of the ability of cancer cells to promote GSH synthesis[30] due to its interaction with a glutamate-cystine transporter xCT.[17] CD44v controls the cell redox potential of TISCs, thus increasing their resistance to induction of ROS and maintaining CSC survival.[15,16] Important cross-talk was recently identified between the Keap1–Nrf2 system and the autophagy-adaptor protein p62–SQSTM1, which was found to compete with Keap1 for binding with Nrf2 by using the STGE motif, and through this competition promoting the stabilization of Nrf2 and upregulation of Nrf2 activity.[31,32] The present findings supported this idea and furthermore suggested the participation of CD44v9 in this pathway. Another protein that can bind to Nrf2 or Keap1, thereby disrupting their interaction is p21$^{WAF1/Cip1}$, which may act together with p62-SQSTM1.[28] It was suggested that high ROS levels are generally detrimental to cells but they also may constitute a barrier to tumorigenesis.[33] The persistent high levels of inflammation and ROS generated by HCV in the liver could result in the development of defense mechanisms to survive in the conditions of persistent oxidative stress, thus p62-SQSTM1 and Nrf2 factors could be activated and drove the CD44v9 expression.

Tumor-initiating stem-like cells were recently suggested to feature enhanced mechanisms of protection from stress induced by ROS that render them resistant to chemotherapy and radiotherapy. As it is accepted that during tumor progression cancer cells are often exposed to high levels of ROS, the ability to escape the consequences of ROS exposure is extremely important for cancer cells to survive and propagate. Tumor-initiating stem-like cells, in which defense against ROS is enhanced by CD44v, are thus thought to drive tumor growth, chemoresistance, and metastasis.[6] Our findings further supported previously published results, where xCT inhibition was shown to selectively induce apoptosis in CD44v-expressing tumor cells without affecting CD44v$^-$ differentiated cells in the same squamous cell carcinomas,[16] and that ablation of CD44 induced loss of xCT from the cell surface and suppressed tumor growth in a transgenic mouse model of gastric cancer.[17]

In our study, the focal pattern of CD44v9 expression and the reverse correlation with cell proliferation markers such as Ki67 and P-p38 suggested that CD44v9 could be a marker of hepatic TISCs. Previously, inhibition of cell proliferation in terms of BrdU labeling was reported in expanded CD44$^+$ glandular elements in gastric tumors of K19-Wnt1/C2 mE mice.[34] Moreover, ablation of CD44v was shown to induce activation of p38^{MAPK}, a downstream target of ROS, and expression of the gene for the cell cycle inhibitor p21$^{WAF1/Cip1}$.[17] In the present study, the poorly differentiated HCCs contained higher percentages of CD44v9$^+$ cells, suggesting that CD44v9 expression is mainly correlated with poor HCC differentiation, which is likely to become the best marker of tumor metastasizing capacity. Hepatocellular carcinomas contained both CD44v9$^+$ and CD44v9$^-$ regions; the CD44v9$^+$ cells that comprised only a small part of the tumor are likely to have high invasion activity, whereas the CD44v9$^-$ cells, which are the majority, have higher proliferation levels. Our data are in good correlation with previous results, indicating that in CD44v9$^+$ cells proliferation could be inhibited through the antioxidant response element/Nrf2/glutathione pathway, characterized by development of resistance to oxidative stress and damage.[17] In addition, ROS have been shown to induce the proliferation of cells that are not resistant to oxidative stress.[23] In addition, actively proliferating cells are considered to be more sensitive to treatment compared to those that are not proliferating and could remain in a "sleeping state" for a long time.[34]

It is conceivable that CD44v9$^+$ HCC overexpression of β-catenin, enzymes involved in energy metabolism, ECM proteins controlled by TGFB1, vimentin, actin cytoskeleton members, and those involved in DNA repair of double-strand breaks and oxidative base modifications, are likely to be mediated by Nrf2, TGFB1, epidermal growth factor receptor, platelet-derived growth factor, basic fibroblast growth factor 2, endothelial growth factor A, IL1A, IL1B, and other factors, which may explain lowered apoptosis, survival maintenance, migration, and invasion activity of CD44v9$^+$ TISCs. In line with our data, clinically, overexpression of CD44s was shown to be associated with high expression of vimentin, low expression of E-cadherin, a high percentage of phospho-Smad2-positive nuclei, mesenchymal spindle-like morphology, tumor invasiveness, and poor prognosis in HCC patients, and reduced disease-free and overall survival. Thus, CD44s are likely to play a critical role in the TGF-β-mediated mesenchymal phenotype.[24]

In conclusion, in the present study, focal CD44v9 overexpression was found in human and CD44v8-10 in mouse HCCs. In human HCV$^+$ HCC cases it was correlated with poorer overall and recurrence-free survival and clinicopathological factors including younger age, a poorly differentiated invasive phenotype of HCC, and increase of ALP levels in the blood, thus suggesting that CD44v9 could be a novel biomarker of liver TISCs and a prognostic factor for HCV$^+$ HCC patients.

Acknowledgments

We thank Kaori Nakakubo, Azusa Inagaki, Keiko Sakata, Yuko Hisabayashi, and Rie Onodera for their valuable technical assistance. This research was supported by grants from the Ministry of Health, Labor and Welfare of Japan and the Japan Science and Technology Corporation.

Disclosure Statement

The authors have no conflict of interest.

Abbreviations

ABC	avidin–biotin complex
ALP	alkaline phosphatase
CD44v	CD44 variant isoform
CSC	cancer stem-like cell
DEN	diethylnitrosamine
HCA	hepatocellular adenoma

HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HR	hazard ratio
IHC	immunohistochemistry
IL1A	interleukin-α
IL1B	interleukin-β
IPA	Ingenuity pathway analysis
Keap1	Kelch-like ECH-associated protein 1
LC	liquid chromatography
NQO1	NAD(P)H quinone oxidoreductase
Nrf2	nuclear factor (erythroid-derived 2)-like 2
P-p38	phospho-p38^{MAPK}
PXR	pregnane X receptor (PXR) ligand–PXR–retinoic acid–retinoid receptor α (RXRα)
ROS	reactive oxygen species
SOD2	superoxide dismutase 2
SQSTM1	sequestosome 1
TGF-β	transforming growth factor-β
TGFB1	transforming growth factor-β1
TISC	tumor-initiating stem-like cell

References

1 Mima K, Okabe H, Ishimoto T *et al.* The expression levels of CD44v6 are correlated with the invasiveness of hepatocellular carcinoma in vitro, but do not appear to be clinically significant. *Oncol Lett* 2012; **3**: 1047–51.
2 Pang RW, Poon RT. Cancer stem cell as a potential therapeutic target in hepatocellular carcinoma. *Curr Cancer Drug Targets* 2012; **12**: 1081–94.
3 Ma S. Biology and clinical implications of CD133(+) liver cancer stem cells. *Exp Cell Res* 2013; **319**: 126–32.
4 Shen Y, Cao D. Hepatocellular carcinoma stem cells: origins and roles in hepatocarcinogenesis and disease progression. *Front Biosci (Elite Ed)* 2012; **4**: 1157–69.
5 Nagano O, Saya H. Mechanism and biological significance of CD44 cleavage. *Cancer Sci* 2004; **95**: 930–5.
6 Nagano O, Okazaki S, Saya H. Redox regulation in stem-like cancer cells by CD44 variant isoforms. *Oncogene* 2013; **32**: 5191–8.
7 Biddle A, Gammon L, Fazil B, Mackenzie IC. CD44 staining of cancer stem-like cells is influenced by down-regulation of CD44 variant isoforms and up-regulation of the standard CD44 isoform in the population of cells that have undergone epithelial-to-mesenchymal transition. *PLoS ONE* 2013; **8**: e57314.
8 Saya H. Molecular mechanisms of brain tumor invasion. *Nihon Rinsho* 2005; **63**(Suppl 9): 61–7.
9 Saya H, Nagano O. Role of CD44-mediated signal in cancer invasion and metastasis. *Tanpakushitsu Kakusan Koso* 2008; **53**: 1225–30.
10 Sugahara KN, Murai T, Nishinakamura H, Kawashima H, Saya H, Miyasaka M. Hyaluronan oligosaccharides induce CD44 cleavage and promote cell migration in CD44-expressing tumor cells. *J Biol Chem* 2003; **278**: 32259–65.
11 Stauder R, Eisterer W, Thaler J, Gunthert U. CD44 variant isoforms in non-Hodgkin's lymphoma: a new independent prognostic factor. *Blood* 1995; **85**: 2885–99.
12 Gotte M, Yip GW. Heparanase, hyaluronan, and CD44 in cancers: a breast carcinoma perspective. *Cancer Res* 2006; **66**: 10233–7.
13 Kainz C, Kohlberger P, Tempfer C *et al.* Prognostic value of CD44 splice variants in human stage III cervical cancer. *Eur J Cancer* 1995; **31A**: 1706–9.
14 Wielenga VJ, van der Neut R, Offerhaus GJ, Pals ST. CD44 glycoproteins in colorectal cancer: expression, function, and prognostic value. *Adv Cancer Res* 2000; **77**: 169–87.
15 Tsugawa H, Suzuki H, Saya H *et al.* Reactive oxygen species-induced autophagic degradation of Helicobacter pylori CagA is specifically suppressed in cancer stem-like cells. *Cell Host Microbe* 2012; **12**: 764–77.
16 Yoshikawa M, Tsuchihashi K, Ishimoto T *et al.* xCT inhibition depletes CD44v-expressing tumor cells that are resistant to EGFR-targeted therapy in head and neck squamous cell carcinoma. *Cancer Res* 2013; **73**: 1855–66.
17 Ishimoto T, Nagano O, Yae T *et al.* CD44 variant regulates redox status in cancer cells by stabilizing the xCT subunit of system xc(-) and thereby promotes tumor growth. *Cancer Cell* 2011; **19**: 387–400.
18 Hirata K, Suzuki H, Imaeda H *et al.* CD44 variant 9 expression in primary early gastric cancer as a predictive marker for recurrence. *Br J Cancer* 2013; **109**: 379–86.
19 Wada T, Ishimoto T, Seishima R *et al.* Functional role of CD44v-xCT system in the development of spasmolytic polypeptide-expressing metaplasia. *Cancer Sci* 2013; **104**: 1323–9.
20 Minagawa M, Ikai I, Matsuyama Y, Yamaoka Y, Makuuchi M. Staging of hepatocellular carcinoma: assessment of the Japanese TNM and AJCC/UICC TNM systems in a cohort of 13,772 patients in Japan. *Ann Surg* 2007; **245**: 909–22.
21 Vauthey JN, Lauwers GY, Esnaola NF *et al.* Simplified staging for hepatocellular carcinoma. *J Clin Oncol* 2002; **20**: 1527–36.
22 Liver Cancer Study Group of Japan. General Rules for the Clinical and Pathological Study of Primary Liver Cancer. The 5th edition (Serial online). [Cited 2009.]
23 Kinoshita A, Wanibuchi H, Imaoka S *et al.* Formation of 8-hydroxydeoxyguanosine and cell-cycle arrest in the rat liver via generation of oxidative stress by phenobarbital: association with expression profiles of p21(WAF1/Cip1), cyclin D1 and Ogg1. *Carcinogenesis* 2002; **23**: 341–9.
24 Mima K, Okabe H, Ishimoto T *et al.* CD44s regulates the TGF-beta-mediated mesenchymal phenotype and is associated with poor prognosis in patients with hepatocellular carcinoma. *Cancer Res* 2012; **72**: 3414–23.
25 Kakehashi A, Ishii N, Shibata T *et al.* Mitochondrial prohibitins and septin 9 are implicated in the onset of rat hepatocarcinogenesis. *Toxicol Sci* 2011; **119**: 61–72.
26 Sasaki JI, Tanabe KK, Takahashi K *et al.* Expression of CD44 splicing isoforms in lung cancers: dominant expression of CD44v8-10 in non-small cell lung carcinomas. *Int J Oncol* 1998; **12**: 525–33.
27 Mitsuishi Y, Motohashi H, Yamamoto M. The Keap1-Nrf2 system in cancers: stress response and anabolic metabolism. *Front Oncol* 2012; **2**: 200.
28 Kiyosawa N, Kwekel JC, Burgoon LD *et al.* Species-specific regulation of PXR/CAR/ER-target genes in the mouse and rat liver elicited by o, p'-DDT. *BMC Genom* 2008; **9**: 487.
29 Maglich JM, Stoltz CM, Goodwin B, Hawkins-Brown D, Moore JT, Kliewer SA. Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. *Mol Pharmacol* 2002; **62**: 638–46.
30 Tanabe KK, Ellis LM, Saya H. Expression of CD44R1 adhesion molecule in colon carcinomas and metastases. *Lancet* 1993; **341**: 725–6.
31 Komatsu M, Kurokawa H, Waguri S *et al.* The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat Cell Biol* 2010; **12**: 213–23.
32 Suzuki T, Yamamoto M. Molecular basis of the Keap1-Nrf2 system. *Free Radic Biol Med* 2015; **88**: 93–100.
33 Gorrini C, Harris IS, Mak TW. Modulation of oxidative stress as an anticancer strategy. *Nat Rev Drug Discov* 2013; **12**: 931–47.
34 Ishimoto T, Oshima H, Oshima M *et al.* CD44+ slow-cycling tumor cell expansion is triggered by cooperative actions of Wnt and prostaglandin E2 in gastric tumorigenesis. *Cancer Sci* 2010; **101**: 673–8.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Immunohistochemical scores of CD44 variant 9 in hepatitis C virus-positive patients with hepatocellular carcinoma.

Table S1. Patient clinicopathological characteristics, tumor characteristics, and treatment characteristics for hepatitis C virus-positive patients with hepatocellular carcinoma (n = 90).

Table S2. Ingenuity pathway analysis of altered molecular functions in CD44 variant 9 $(CD44v9)^+$ and $CD44v9^-$ hepatocellular carcinomas.

Table S3. Ingenuity canonical pathway analysis in CD44 variant 9 $(CD44v9)^+$ and $CD44v9^-$ hepatocellular carcinomas.

Table S4. Ingenuity pathway analysis of upstream regulators in CD44 variant 9 $(CD44v9)^+$ and $CD44v9^-$ hepatocellular carcinomas.