

High CD44s expression is associated with the EMT expression profile and intrahepatic dissemination of hepatocellular carcinoma after local ablation therapy

Kosuke Mima · Hiromitsu Hayashi · Katsunori Imai · Hideyuki Kuroki · Shigeaki Nakagawa · Hirohisa Okabe · Akira Chikamoto · Masayuki Watanabe · Toru Beppu · Hideo Baba

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Abstract

Background/purpose Local ablation therapy (LAT) is a widely used treatment for hepatocellular carcinoma (HCC) because it is less invasive than hepatic resection. The precise molecular mechanism underlying local HCC recurrence after LAT is largely unknown. The CD44 standard isoform (CD44s) is involved in epithelial–mesenchymal transition (EMT) in HCC. We investigate the significance of CD44s expression and EMT expression profile in local HCC recurrence after LAT.

Methods We studied the expression levels of CD44s, EMT expression profile (E-cadherin^{low}/vimentin^{high} expression) and their association with clinicopathological factors in 30 HCC samples from patients with locally recurrent HCCs after LAT following hepatic resection. The alterations of CD44s expression was compared with those in initial HCCs from 150 patients without prior any anticancer treatment including LAT.

Results A high CD44s expression was significantly associated with the EMT expression profile ($P = 0.002$), and it was also detected with a higher frequency in the

locally recurrent HCCs after LAT compared to initial HCCs ($P < 0.001$). In addition, high CD44s expression was associated with the intrahepatic dissemination of HCC after LAT ($P = 0.006$).

Conclusions These results suggest that high CD44s expression is associated with the aggressive recurrence pattern via EMT after LAT for HCC.

Keywords CD44s · EMT · HCC · Intrahepatic dissemination · Local ablation therapy

Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent and the third most deadly type of cancer, diagnosed in more than half a million people worldwide each year [1]. Surgical resection and liver transplantation can be curative for early-stage HCC, but the resectability is limited in multifocal disease because of the poor liver functional reserve and the invasiveness of these procedures [2]. As an alternative modality in such cases, local ablation therapies (LAT),—which include percutaneous ethanol injection therapy (PEIT), microwave coagulation therapy (MCT), and radiofrequency ablation (RFA)—have often been performed in patients with HCC because of their relatively low invasiveness [3]. However, LAT shows a relatively high post-treatment recurrence rate. Although the intrahepatic recurrence rate after LAT for HCC, including ablation site recurrence, is 29–65 % [4], the precise molecular mechanism underlying HCC recurrence after LAT still remains largely unknown. The identification of new molecular mechanisms for HCC recurrence after LAT is, therefore, of great importance and remains an urgent need for improving outcome for patients with HCC.

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K. Mima · H. Hayashi · K. Imai · H. Kuroki · S. Nakagawa · H. Okabe · A. Chikamoto · M. Watanabe · T. Beppu · H. Baba (✉)
Department of Gastroenterological Surgery,
Graduate School of Medical Sciences, Kumamoto University,
1-1-1 Honjo, Kumamoto 860-8556, Japan
e-mail: hdobaba@kumamoto-u.ac.jp

Epithelial–mesenchymal transition (EMT) is a pivotal mechanism of cancer invasion and metastasis, as epithelial cells lose their polarity and acquire the migratory properties of mesenchymal cells. The characteristic changes that occur during EMT include the downregulation of epithelial markers such as E-cadherin, and upregulation of mesenchymal markers such as vimentin [5]. Some types of cancers, including breast and colon cancers, show an association between the EMT expression profiles and tumor recurrence [6, 7]. CD44, a major adhesion molecule of the extracellular matrix, has been implicated in a wide variety of physiological processes, including leukocyte homing and activation, wound healing and cell migration [8, 9]. The cells produce CD44 protein isoforms through alternative mRNA splicing. We have previously shown that the CD44 standard isoform (CD44s) regulates mesenchymal phenotypes through EMT and increases tumor cell invasion in HCC [10]. Based on the previous evidence, we investigate the significance of CD44s expression and EMT expression profile in local HCC recurrence after LAT.

Materials and methods

Patients and treatment

The indications for LAT, including PEIT, MCT, and RFA, for HCC are: tumor size ≤ 5 cm; ≤ 3 tumors; no portal vein tumor thrombus; tumor not adjacent to major vessels; no uncontrollable ascites; total-bilirubin levels ≤ 3 mg/dl; and prothrombin activity ≥ 40 %. All patients underwent RFA by hepatobiliary surgeons. Complete ablation of a tumor smaller than 1.5 cm in diameter can be accomplished with single puncture, and multiple overlapping ablations are performed to obtain complete destruction of a tumor larger than 2 cm in diameter [11].

Hepatic resection is indicated for local HCC recurrence after LAT despite a sufficient margin. Between April 1992 and November 2011, 30 patients with HCC underwent hepatic resection because of local HCC recurrence after complete LAT in the Department of Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University. All of these patients were histologically proven hepatocellular carcinoma and enrolled in this study to assess the clinical significance of the expression levels of CD44s and EMT markers using the resected liver specimens of locally recurrent HCCs after complete LAT. Nine of these 30 patients were diagnosed with highly invasive recurrence with intrahepatic dissemination of HCC, which was defined as a multinodular or diffuse recurrence beyond the ablation site [12]. The remaining 21 patients had localized solitary recurrences. We have previously analyzed the expression

of CD44s, E-cadherin, and vimentin in the initial HCCs which were the resected liver specimens from 150 patients who did not receive any prior anticancer treatment including LAT [10]. To assess the alterations of the CD44s expression and the EMT expression profile, we compared these expression levels in the locally recurrent HCCs after LAT and the initial HCCs. The pathological diagnoses and clinicopathological factors were established using general guidelines for primary liver cancer of the Liver Cancer Study Group of Japan [13, 14] and the American Joint Committee on Cancer/International Union Against Cancer staging systems [15]. This study was approved by the Human Ethics Review Committee of the Graduate School of Medicine, Kumamoto University (Kumamoto, Japan).

Immunohistochemistry and scoring

Sample processing and immunohistochemistry (IHC) procedures were performed as described in a previous report [10]. The endogenous peroxidase activity was blocked using 3 % hydrogen peroxide and sections were incubated with diluted antibodies. Subsequent reactions were performed with a biotin-free horseradish peroxidase enzyme-labeled polymer (Envision Plus Detection System, Dako Co.). Positive reactions were visualized by adding diaminobenzidine solution, and then counterstaining with Mayer's hematoxylin. Primary antibodies for E-cadherin (1:100 dilution; BD), vimentin (1:50 dilution; Santa Cruz Biotechnology), and CD44s (1:300 dilution; Bender MedSystems) were used. All IHC staining results were independently scored by two pathologists. The expression levels and the localization of CD44s, E-cadherin, and vimentin of cancer cells in the tumors were evaluated. The membranous E-cadherin, cytoplasmic vimentin, and membranous CD44s expression levels in cancer cells of the tumors were interpreted using guidelines published in previous studies [10]. For membranous E-cadherin and CD44s, and cytoplasmic vimentin, 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. For membranous E-cadherin, 2+ and 3+ samples were defined as high expression. For cytoplasmic vimentin, and membranous CD44s, 3+ specimens were defined as high expression.

Statistical analyses

Categorical variables were compared using the χ^2 test. The statistical analyses were performed as indicated with a statistical analysis software program (Excel Statistics, Social Survey Research Information Co.). Differences were considered to be significant when $P < 0.05$.

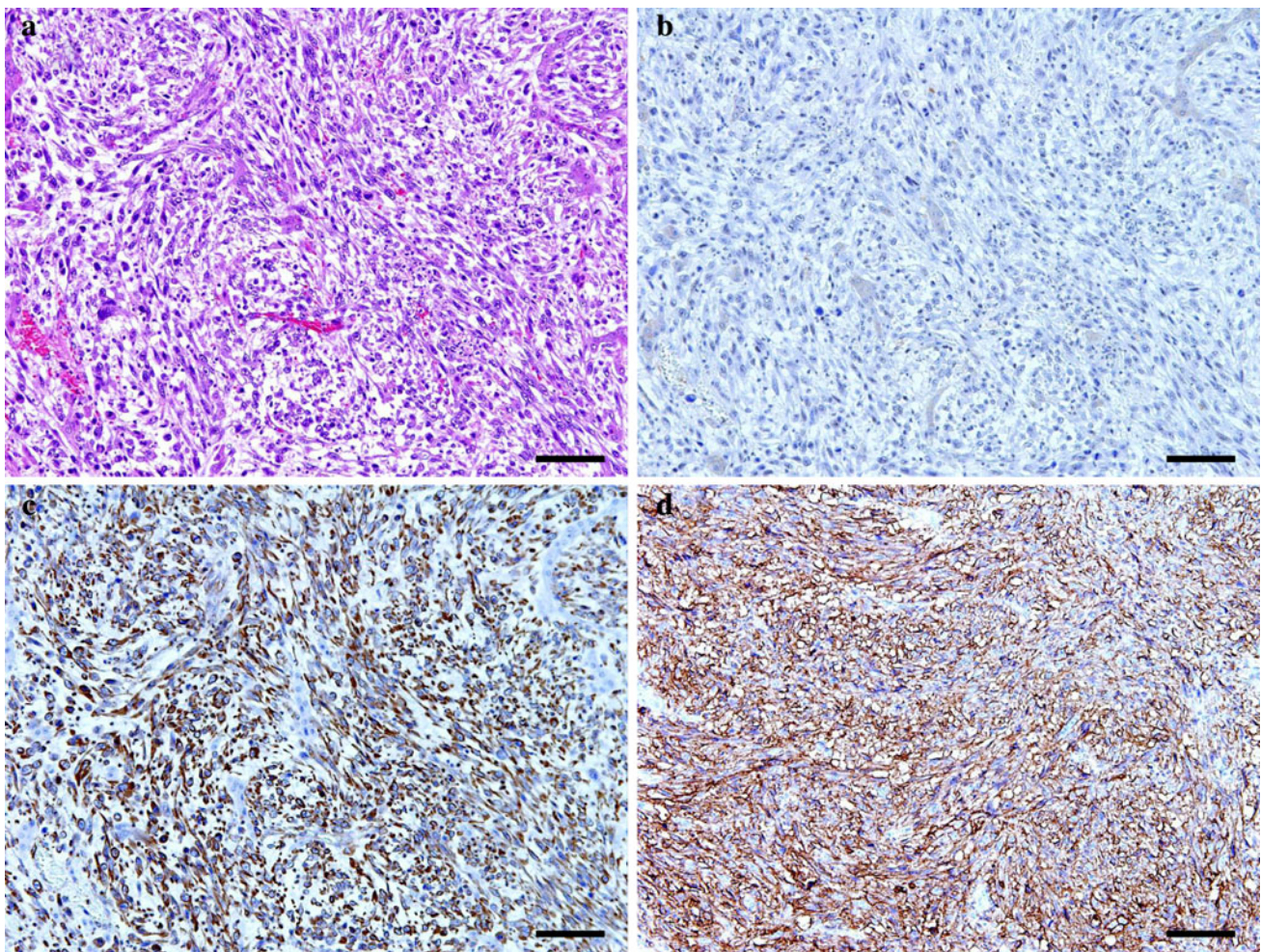


Fig. 1 Immunohistochemical staining of E-cadherin, vimentin, and CD44s in locally recurrent HCCs after LAT. A representative case (a H&E staining) shows low E-cadherin expression (b), high vimentin expression (c) and high CD44s expression (d). Scale bars 100 μm

Results

High CD44s expression is associated with the EMT expression profile in locally recurrent HCCs after LAT

To assess the association between local HCC recurrence after LAT and the expression of CD44s/EMT, we analyzed the expression levels of CD44s, E-cadherin (an epithelial marker), and vimentin (a mesenchymal marker) in 30 samples from patients with locally recurrent HCCs after LAT using IHC. High CD44s expression and the EMT expression profile (E-cadherin^{low}/vimentin^{high} expression) were detected in 63.3 % (19/30) and 36.7 % (11/30) of the samples, respectively (Fig. 1). A high expression of CD44s was significantly associated with the EMT expression profile in locally recurrent HCCs after LAT ($P = 0.002$; Table 1). It is currently difficult to obtain samples from patients without local HCC recurrence after LAT, because a needle biopsy prior to LAT can lead to extrahepatic

Table 1 Correlation between the expression of CD44s and the E-cadherin/vimentin expression profiles in the local recurrence after local therapy for HCC patients

	CD44s low expression (n = 11)	CD44s high expression (n = 19)	P value*
E-cadherin ^{low} /vimentin ^{high} expression profile			
Negative (n = 19)	11	8	0.002
Positive (n = 11)	0	11	

CD44s CD44 standard isoform

* Estimated by the χ^2 test

tumor seeding [16]. We therefore compared the expression of CD44s and EMT markers between the locally recurrent HCCs after LAT and initial HCCs obtained from 150 patients who did not receive any preoperative anticancer

treatment, which theoretically represent similar expression profiles as would be seen in the tumor samples that would be obtained by needle biopsy prior to LAT. In the locally recurrent HCCs, the detection of multiple tumors, an invasive macroscopic appearance, poor tumor differentiation, high CD44s expression, and the EMT expression profile was significantly higher than in the initial HCCs, although there were no significant differences in the AFP and PIVKA-II levels, or the vascular invasion (Table 2).

High CD44s expression is associated with intrahepatic dissemination of HCC in patients with local recurrence after LAT

We next investigated the association between CD44s expression in the local HCC recurrence and clinicopathological factors, including an aggressive recurrence pattern, such as the intrahepatic dissemination of HCC leading to fatal outcomes after LAT [12]. A significant correlation was seen between high CD44s expression and an invasive macroscopic appearance ($P = 0.044$), and the intrahepatic dissemination of HCC ($P = 0.006$) (Table 3). High CD44s expression showed a stronger association with a large tumor size (57.9–27.3 %, $P = 0.105$) and frequent vascular invasion (21.1–0 %, $P = 0.102$) than did low CD44s

expression, although the difference was not statistically significant. The EMT expression profile was significantly associated with the invasive macroscopic appearance ($P = 0.009$), the early recurrence after LAT ($P = 0.029$), and the intrahepatic dissemination of HCC ($P = 0.026$) (Supplementary Table S1).

Discussion

We found that high CD44s expression was associated with the EMT expression profile and aggressive recurrence, such as the intrahepatic dissemination of HCC. In initial HCCs obtained by hepatic resection, high CD44 expression was positively associated with the EMT expression profile [10]. A similar association between CD44s and the EMT expression profile was found in the locally recurrent HCCs after LAT. To investigate whether CD44s and EMT are involved in local HCC recurrence after LAT, the ideal way to determine the role of CD44s in local recurrence after LAT would be compare the expression of CD44s and EMT expression profiles between the HCC patients with and without local recurrence after LAT. Because of the risk of extrahepatic tumor seedings by a needle biopsy prior to LAT [16], we could not obtain tumor samples from patients without local recurrence after LAT. However, in this study,

Table 2 Correlation between the initial HCCs and the locally recurrent HCCs after local ablation therapy of clinicopathological factors

	Initial HCCs ($n = 150$)	Locally recurrent HCCs ($n = 30$)	P value*
Age (≤ 60 / > 60), years	44/106	7/23	0.606
Sex (male/female)	123/27	26/4	0.537
HBs-Ag (negative/positive)	107/43	18/12	0.219
HCV-Ab (negative/positive)	81/69	16/14	0.947
Child–Pugh classification (A/B)	135/15	29/1	0.242
AFP (≤ 20 / > 20), ng/ml	76/74	17/13	0.548
PIVKA-II (≤ 91 / > 91), mAU/ml	73/77	16/14	0.641
Tumor size (≤ 3 / > 3), cm	50/100	16/14	0.038
Tumor number (1/ ≥ 2)	106/44	12/18	0.001
Macroscopic appearance simple (nodular type/other)	99/51	12/18	0.008
Tumor differentiation (moderate, well/poor)	121/29	18/12	0.014
LCSGJ TNM stage (1, 2/3, 4)	89/61	8/22	0.001
AJCC/UICC TNM stage (1, 2/3, 4)	122/28	25/5	0.796
Vascular invasion (absent/present)	132/18	26/4	0.839
EMT expression profile (negative/positive)	129/21	19/11	0.003
CD44s expression (low/high)	113/37	11/19	<0.001

Bold text denotes a statistically significant difference

HCC hepatocellular carcinoma, HBs-Ag hepatitis B surface antigen, HCV-Ab hepatitis C antibody, AFP α -fetoprotein, PIVKA II protein-induced vitamin K absence-II, LCSGJ Liver Cancer Study Group of Japan, AJCC/UICC American Joint Committee on Cancer/International Union Against Cancer, EMT epithelial–mesenchymal transition, CD44s CD44 standard isoform

* Estimated by the χ^2 test. Vascular invasion, portal vein (3rd branch, 2nd branch, 1st branch, or trunk) or hepatic vein (trunk of hepatic vein or IVC) invasion were defined via macroscopic examination of the resected specimens. The TNM stage was established using both the LCSGJ 5th Revised Version TNM classification and AJCC/UICC 7th TNM staging system

Table 3 Correlation between the CD44s expression in the local HCC recurrence after local ablation therapy and the clinicopathological factors

	CD44s low expression (n = 11)	CD44s high expression (n = 19)	P value*
Age (≤ 60 / > 60), years	3/8	4/15	0.698
Sex (male/female)	10/1	16/3	0.603
HBs-Ag (negative/positive)	9/2	9/10	0.063
HCV-Ab (negative/positive)	4/7	12/7	0.156
Child–Pugh classification (A/B)	11/0	18/1	0.439
AFP (≤ 20 / > 20), ng/ml	8/3	9/10	0.177
PIVKA-II (≤ 91 / > 91), mAU/ml	7/4	8/11	0.256
Tumor size (≤ 3 / > 3), cm	8/3	8/11	0.105
Tumor number ($1 \geq 2$)	4/7	8/11	0.757
Macroscopic appearance (simple nodular type/other)	7/4	5/14	0.044
Tumor differentiation (moderate, well/poor)	8/3	10/9	0.279
LCSGJ TNM stage (1, 2/3, 4)	5/6	3/16	0.077
AJCC/UICC TNM stage (1, 2/3, 4)	10/1	15/4	0.397
Prior local therapy (PEIT/MCT or RFA)	3/8	5/14	0.955
Time to local recurrence (< 3 months/ > 3 months)	2/9	5/14	0.612
Vascular invasion (absent/present)	11/0	15/4	0.102
Intrahepatic dissemination of HCC (absent/present)	11/0	10/9	0.006
Extrahepatic recurrence (absent/present)	9/2	16/3	0.866

Bold text denotes a statistically significant difference

HCC hepatocellular carcinoma, CD44s CD44 standard isoform, HBs-Ag hepatitis B surface antigen, HCV-Ab hepatitis C antibody, AFP α -fetoprotein, PIVKA II protein-induced vitamin K absence-II, LCSGJ Liver Cancer Study Group of Japan, AJCC/UICC American Joint Committee on Cancer/International Union Against Cancer, PEIT percutaneous ethanol injection therapy, MCT microwave coagulation therapy, RFA radiofrequency ablation

* Estimated by the χ^2 test. Vascular invasion, portal vein (3rd branch, 2nd branch, 1st branch, or trunk) or hepatic vein (trunk of hepatic vein or IVC) invasion were defined via macroscopic examination of the resected specimens. The TNM stage was established using both the LCSGJ 5th Revised Version TNM classification and AJCC/UICC 7th TNM staging system

the locally recurrent HCCs after LAT were characterized by a higher CD44s expression and a stronger EMT expression profile compared with initial HCCs, although there were no significant differences in the AFP and PIVKA-II levels, or the vascular invasion, which are reported to be associated with the high malignant potential of HCC. These results suggest that CD44s may play a role in local HCC recurrence after LAT via EMT.

The cause of the intrahepatic dissemination of HCC after LAT, which leads to fatal outcomes, remains largely unknown [12]. Remarkably, high CD44s expression was associated with the intrahepatic dissemination of HCC after LAT in this study. To the best of our knowledge, the present study is the first to describe the association between CD44s expression, the EMT expression profile and this aggressive recurrence pattern. In the current study, the pathological diagnoses and clinicopathological factors were established using both the LCSGJ 5th Revised Version TNM classification and AJCC/UICC 7th TNM staging system. The major difference between the LCSGJ and the AJCC/UICC TNM stage is the cutoff value for tumor size, which are 2 cm and 5 cm, respectively. This difference in

the TNM staging system may have contributed to the statistical difference between the LCSGJ and the AJCC/UICC TNM stage in the clinicopathological factors of initial HCCs versus those of the locally recurrent, and in the CD44s expression.

Little is known about the molecular mechanisms underlying HCC recurrence after LAT. The pretreatment serum level of VEGF, which is a critical mediator of angiogenesis in HCC, is an independent unfavorable prognostic factor in HCC patients treated with RFA, thus suggesting that angiogenesis is involved in the recurrence after LAT for HCC [17]. Keratin 19-positive HCC, which may originate from hepatic progenitor cells, is related to a high recurrence rate after RFA [18]. A high serum level of hyaluronic acid, which is an essential component of the extracellular matrix, and hepatitis B viral infection are the main prognostic factors for local recurrence after RFA for HBV-related HCC [19]. Our study therefore provides new insight into the molecular mechanism by which CD44s may mediate aggressive recurrence patterns after LAT for HCC. The molecular mechanism and causal relationship underlying the high CD44s expression and EMT

expression profile in locally recurrent HCCs remains unclear from our study. However, we have previously shown that TGF- β signaling in HCC cells is a potent inducer of mesenchymal phenotypes via the induction of CD44s expression [10]. LAT also induces such cytokines as interleukin (IL)-1 β and IL-6 [20], which might contribute to CD44s expression. Further studies are needed to investigate these mechanisms.

In conclusion, we found that high CD44s expression was associated with the EMT expression profile and the intrahepatic dissemination of HCC after LAT. These results suggest that high CD44s expression is associated with the aggressive recurrence pattern via EMT after LAT for HCC.

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Conflict of interest The authors declare that they have no conflict of interest.

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