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Original Article

The relationship between the tumor microenvironment of hepatocellular carcinoma - including cancer-associated fibroblasts and tumor-associated macrophages - and apparent diffusion coefficient

Yu Saito, Yuji Morine, Shinichiro Yamada, Hiroki Teraoku, Katsuki Miyazaki, Tetsuya Ikemoto, Mitsuo Shimada

Department of Surgery, Tokushima University, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan

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Abstract: The tumor microenvironment is critical for the acquisition of tumor malignancy in various cancer types. The objectives of this study were to investigate whether the levels of cancer-associated fibroblasts (CAFs) and tumor-associated macrophages (TAMs) reflect the prognosis of patients with hepatocellular carcinoma (HCC) after hepatectomy (Hx) and to determine whether the apparent diffusion coefficient (ADC) from diffusion-weighted imaging (DWI) reflects CAF and TAM expression. The study cohort comprised 109 patients who underwent initial curative resection for HCC. Alpha smooth muscle actin (αSMA) was selected as a CAF marker and CD204 as a TAM marker. Protein expression was immunohistochemically evaluated in the intratumoral regions of resected specimens. Clinicopathological factors, including the long-term prognosis after Hx, were investigated between αSMA-negative and -positive tumors and between CD204-negative and -positive tumors. The correlation between CAF/TAM marker expression and the calculated minimum ADC using DWI was also evaluated. αSMA-positive expression was correlated with tumor number, invasive growth pattern, and advanced stage. CD204-positive expression was correlated with the presence of venous invasion. Both αSMA-positive expression and CD204-positive expression were significant prognostic factors in the univariate analysis of overall survival and disease-free survival. αSMA/CD204 double positivity was associated with an extremely poor prognosis after Hx and was a significant independent prognostic factor for overall survival (P=0.02, hazard ratio: 3.27). Patients with double positivity also showed a significantly higher ADC^{low} rate (83%). In conclusion, expression of both CAF and TAM markers reflected a poor prognosis after Hx. Furthermore, the preoperative ADC could be a clinical surrogate marker in the tumor microenvironment in patients with HCC.

Keywords: Tumor microenvironment, cancer-associated fibroblast, tumor-associated macrophage, hepatocellular carcinoma, apparent diffusion coefficient

Introduction

Systemic therapy for hepatocellular carcinoma (HCC) has dramatically changed over the past few decades. Immune checkpoint inhibitors, such as anti-programmed death receptor-1, anti-programmed death ligand-1, and anti-cytotoxic T-lymphocyte antigen-4 monoclonal antibodies, have been tested worldwide [1]. Notably, atezolizumab-bevacizumab (IMbrave150 trial) [2] and durvalumab-tremelimumab (HIMALAYA trial) [3] demonstrated superiority over sorafenib, establishing new first-line options. These treatments target various immune cell types in the tumor microenvironment (TME). The TME includes cancer cells, T cells (CD4+, CD8+, and regulatory), B cells, dendritic cells, natural killer cells, neutrophils, myeloid-derived suppressor cells, cancer-associated fibroblasts (CAFs), and tumor-associated macrophages (TAMs) [4-6].

Several reports from our department have focused on CAFs and TAMs in HCC. Lactate derived from cancer cells stimulates TAMs through nuclear factor erythroid 2-related factor 2 (NRF2) activation, and TAMs secrete vascular endothelial growth factor (VEGF) to pro-

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mote cancer cell migration through NRF2-induced epithelial-mesenchymal transition [7]. Cancer cell-derived VEGF also activates TAMs [8]. Interleukin-6 derived from CAFs promotes HCC progression through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway [9], while the B-cell activating factor/nuclear factor kappa B axis in CAFs contributes to sorafenib resistance in HCC [10]. Moreover, the interaction between CAFs and TAMs via the osteopontin pathway [11] and the plasminogen activator inhibitor-1 pathway [12] increases tumor malignancy. Furthermore, TU-100 antagonizes the activation of both CAFs [13] and TAMs [14]. Hence, CAFs and TAMs could be therapeutic targets for HCC. However, because all data were obtained from in vitro or animal experiments, it is necessary to investigate the role of CAFs and TAMs in a clinical setting.

In previous reports using clinical samples, fibroblast activation protein (FAP), which is a CAF marker, was immunohistochemically analyzed in surgically resected specimens, and patients with high FAP expression exhibited a poor prognosis [15]. High α-SMA expression also indicates high CAF infiltration, and such patients also showed a poor prognosis [16]. The TAM markers CD163 [17, 18], CD169 [19], and CD204 [20] were immunohistochemically analyzed, and patients with high TAM numbers showed both a poor prognosis and high CAF numbers. However, there have been no reports investigating both CAF and TAM marker expression in HCC.

Diffusion-weighted imaging (DWI) is a functional magnetic resonance imaging (MRI) technique that can evaluate water molecule diffusion and assess the histopathological condition of tissues and organs using a high scan speed without a contrast agent [21]. The apparent diffusion coefficient (ADC) provides a quantitative measurement of diffusion characteristics [22]. We previously focused on the ADC for prognostic prediction in patients with intrahepatic cholangiocarcinoma (IHCC) [23, 24], colorectal liver metastasis [25], and HCC [26]. Furthermore, the ADC can predict tumor-infiltrating lymphocytes in IHCC tissue [24]. To the best of our knowledge, however, no studies have focused on the prognostic prediction of both CAF and TAM marker expression and its correlation with the ADC.

Therefore, the objective of this study was to investigate whether the CAF and TAM levels reflect the prognosis of patients with HCC after hepatectomy (Hx). We also investigated whether the ADC derived from DWI reflects CAF and TAM expression in the TME.

Materials and methods

Patients

One hundred nine patients with HCC were enrolled in this study among 470 patients with HCC who underwent curative Hx at Tokushima University Hospital from April 2000 to March 2021. The inclusion criteria were primary hepatic resection, no other treatments prior to surgery, no extrahepatic metastasis, pathologically proven HCC, a >1-year follow-up period, and presence of immunohistochemistry (IHC). Tumor status and stage were defined according to the Barcelona Clinic Liver Cancer (BCLC) staging system. Patient background and disease characteristics at baseline, such as age, sex, hepatitis viral infection status, and liver function (including the indocyanine green retention rate at 15 min), were collected from the medical records. Furthermore, tumor factors, including serum tumor markers, maximum tumor diameter, tumor number, and pathological tumor status, were also collected. This study was approved by the Tokushima University Hospital Ethics Committee, and the experiments were performed in accordance with the approval guidelines (Tokushima Clinical Trial Management System Number, 3215-3).

Follow-up after Hx

Monthly follow-up was conducted by assessment of tumor markers [alpha-fetoprotein (AFP), AFP-L3, and des-gamma carboxy prothrombin (DCP)] and ultrasonography. Dynamic computed tomography and gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA)-enhanced MRI were conducted at 3 and 6 months after surgery. We defined recurrence as the appearance of new lesions with radiological features typical of HCC, as confirmed by at least two imaging methods.

IHC

Immunohistochemical analysis was performed in accordance with the protocol used in our department, which has been previously report-

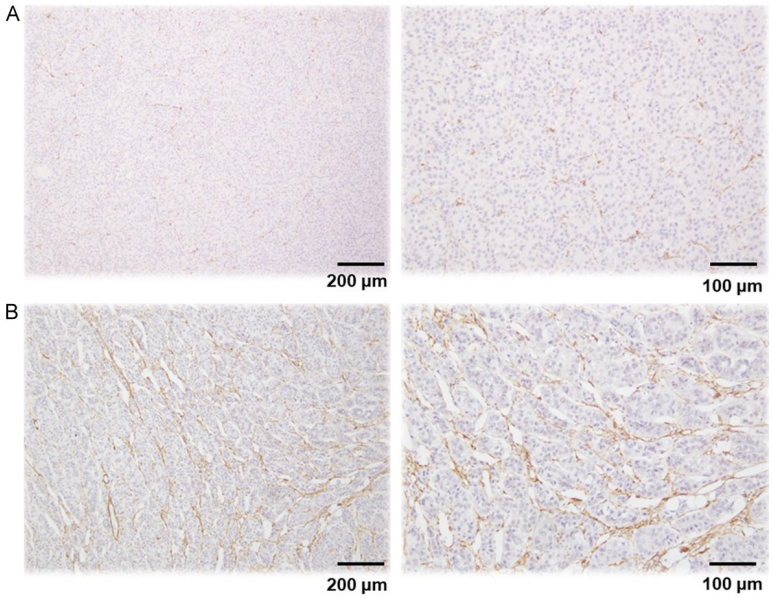

Figure 1. Immunohistochemistry of αSMA. αSMA expression scores were assessed based on both staining area (0, 0%-50%; 1, ≥50% positive area) and intensity (0, weak; 1, mild; 2, strong). A. "Area score 0" and "staining score 0 or 1" were considered to indicate negativity. B. "Area score 1" or "area score 0" and "staining score 2" were considered to indicate positivity.

ed [27]. Briefly, sections were deparaffinized with xylene and then rehydrated in a graded ethanol series. The sections were treated with 3% hydrogen peroxide in methanol for 10 min to quench the endogenous peroxidase activity. Antigen retrieval was performed by boiling in 10 mM citrate buffer (pH 6) using a microwave. After incubation with 1% bovine serum albumin to block nonspecific antibody binding, the sections were incubated with primary antibodies against rabbit monoclonal αSMA antibody (1:100 dilution, ab7817; Abcam, Tokyo, Japan) and CD204 antibody (1:50, KT022; Trans Genic Inc., Fukuoka, Japan) for 60 min at room temperature. After washing with phosphate-buffered saline, the sections were subjected to the Dako REAL EnVision/HRP detection system (Dako Corporation, Tokyo, Japan) for 60 min at room temperature. The peroxidase reaction was developed with 3,3'-diaminobenzidine as the chromogen. The sections were counterstained with 10% Mayer's hematoxylin, dehydrated in a graded ethanol series, treated with xylene, and mounted in synthetic resin.

Evaluation of αSMA and CD204 IHC

αSMA and CD204 expression was quantified by screening the entire tumor area in a low-power field (40× magnification) and randomly evaluating four areas (200× magnification) in the intratumoral region [tumor center more than one high-power field (HPF) away from the tumor margin]. Assessment was performed by two pathologists at our institution who were blinded to information about the patients' clinical backgrounds and prognosis.

αSMA expression scores were assessed based on both the staining area (0, 0%-50% positive area; 1, >50% positive area) and staining intensity (0, weak; 1, mild; 2, strong) [28]. If the area score was 0 and the staining score was 0 or 1, the result was considered negative (**Figure 1A**). If the area score was 1 or the area score was 0 with a staining score of 2, the result was considered positive (**Figure 1B**). Additionally, the number of CD204-positive cells was counted [29]; a mean of <90 positive cells/HPF was considered negative, and a mean of ≥90 positive cells/HPF was considered positive (**Figure 2**). The patients were divided into positive and negative groups for each staining. Finally, the patients were divided into three groups according to the IHC results: αSMA/CD204 double-negative group, αSMA-positive or CD204-positive group, and αSMA/CD204 double-negative group.

ADC calculation

MRI was performed using a 1.5-T superconducting unit (Signa HDe/Explorer; GE Medical Systems, Milwaukee, WI, USA) with an eight-channel phased-array coil. Fast spin-echo T2-weighted imaging and DWI (b=0, 20, and 800 s/mm^2) were also performed. ADC values ($\times 10^{-3}$ mm^2/s) were calculated for tumors within regions of interest using manual tracings from ADC maps using a 3D image analyzer (Synapse Vincent, Fujifilm Medical, Tokyo, Japan) [23]. Synapse Vincent can automatically show mean, minimum, and maximum values from free-form green outlines (**Figure 3**). The minimum ADC was used because our previous reports showed that the minimum ADC predicted patients'

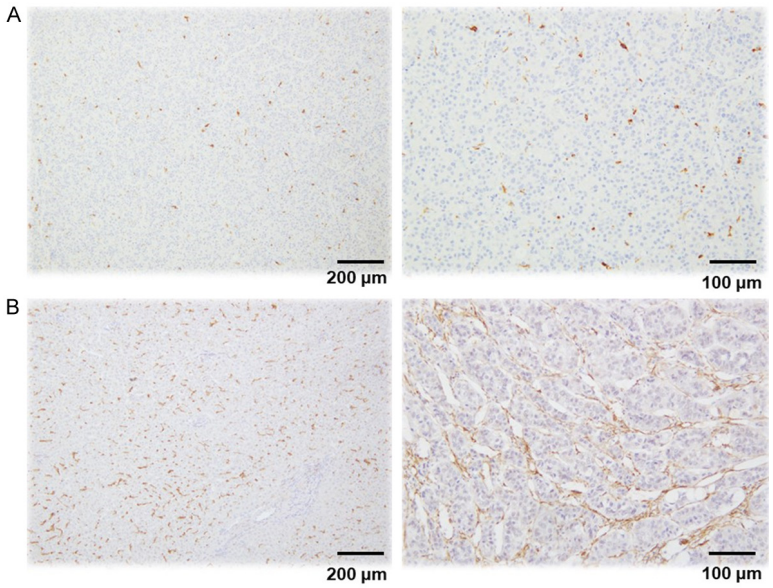

Figure 2. Immunohistochemistry of CD204. For the CD204 staining, positive cells were counted, and "mean positive cells <90 cells/HPF" were considered to indicate negativity (A), while "mean positive cells ≥90 cells/HPF" were considered to indicate positivity (B).

prognosis [23-26]. The cutoff value for the minimum ADC was 0.74×10^{-3} mm^2/s, calculated by a receiver operating characteristic curve for prediction of CAF/TAM double positivity (Figure S1). Sensitivity was calculated as 71% and 1 - specificity as 83%.

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp., Armonk, NY, USA). A *p*-value of <0.05 was considered statistically significant. Relationships between αSMA or CD204 expression and the clinicopathological variables were analyzed using the chi-squared test and Mann-Whitney U test. Survival curves were calculated using the Kaplan-Meier method and compared with the log-rank test. All factors found to be statistically significant by the univariate analysis were included in a multivariate analysis using a Cox proportional hazards model to identify independent factors influencing survival. The factors included in the analysis were sex (male or female), hepatic viral status (hepatitis B surface antigen present, hepatitis C virus antibodies present, or both negative), modified albumin-bilirubin (mALBI) grade (1, 2a/2b, or 3), tumor number (single or multiple), tumor size (<5 or >5 cm), tumor differentiation (well, moderate, or poor), tumor growth type (expansive or invasive growth), portal invasion (absent or present), vessel invasion (absent or present), stage (I, II/III, or IV), BCLC stage (0, A/B, or C), serum AFP level (<400 or >400 ng/mL), and serum DCP level (<500 or >500 mAU/mL). Comparisons of the three groups were analyzed with one-way analysis of variance followed by Fisher's post hoc correction.

Results

Correlation between clinicopathological factors and CAF/TAM markers

αSMA-positive expression was correlated with tumor number (*P*=0.01), invasive growth pattern (*P*=0.03), and advanced stage (*P*<0.01) (**Table 1**). CD204-positive expression was correlated with the presence of venous invasion alone (*P*<0.01); it also tended to be correlated with tumor number (*P*=0.09) (**Table 2**).

Long-term prognosis after Hx

The univariate analysis of overall survival (OS) revealed the following prognostic factors: mALBI grade, tumor number, tumor growth pattern, portal invasion, TNM stage, BCLC stage, serum DCP level, and αSMA and CD204 double-positive expression. The multivariate analysis of OS showed that αSMA and CD204 double-positive expression [*P*=0.03, hazard ratio (HR): 3.27] and the mALBI grade (*P*=0.03, HR: 3.19) were significant independent prognostic factors (**Table 3**).

Univariate analysis of disease-free survival (DFS) revealed the following prognostic factors: mALBI grade, tumor number, tumor growth pattern, TNM stage, BCLC stage, and αSMA and CD204 double-positive expression. The multivariate analysis of DFS showed that the mALBI grade (*P*<0.01, HR: 2.79) was the only significant independent prognostic factor (**Table 4**).

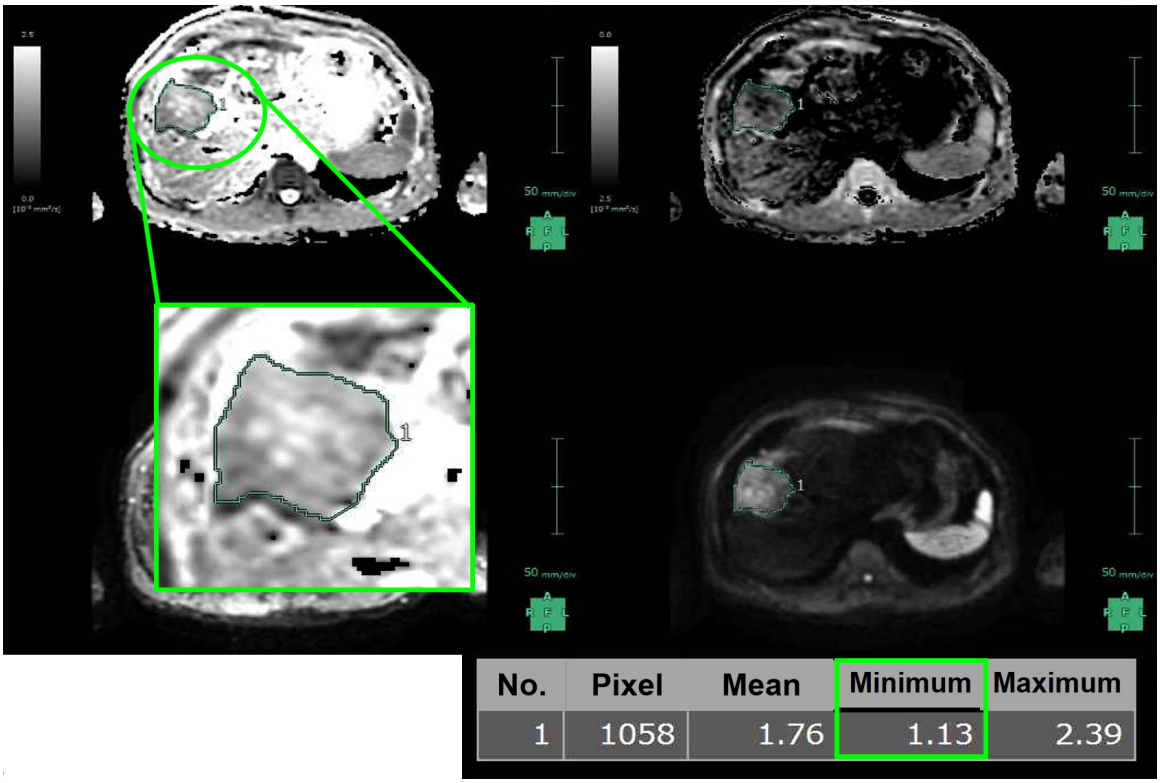

Figure 3. ADC calculation with minimum value. Synapse Vincent can show mean, minimum, and maximum values from free-form green outlines automatically. Minimum ADC values were used because our previously reports showed minimum ADC value predicted patients' prognosis.

CAF and TAM expression as prognostic prediction markers

Patients positive for CAF markers showed a poor prognosis after Hx for both OS (*P*=0.02) and DFS (*P*=0.04) (**Figure 4A**). TAM markers showed a prognostic curve similar to that of CAF markers (OS: *P*=0.04, DFS: P=0.06) (**Figure 4B**). Furthermore, the expression of CAF and TAM markers was positively correlated (*P*=0.04) (**Figure 4C**). When patients were divided into a CAF/TAM double-negative group, a CAF-positive or TAM-positive group, and a CAF/TAM double-negative group, both OS and DFS were clearly stratified by these two markers (**Figure 4D**).

ADC as a clinical surrogate marker in TME

The minimum ADC in the CAF/TAM double-positive group was lower than that in the other groups (double-negative group: 1.06±0.05, positive and negative group: 1.02±0.09, double-positive group: 0.82±0.09; *P*=0.06 for double-negative vs. double-positive group). Using a cutoff value of 0.74×10^{-3} mm^2/s, the CAF/TAM double-positive group showed a significantly higher ADC^{low} rate (double-negative group: 13%, positive and negative group: 19%, and double-positive group: 83%; *P*=0.01) (**Figure 5**).

Discussion

In the present study, the expression of intratumoral CAF and TAM markers was analyzed in resected specimens, and double-positive patients were found to have an extremely poor prognosis after Hx. Furthermore, CAF marker expression was significantly correlated with TAM marker expression. It is critical to analyze not only single markers, but also the combination of CAF and TAM markers for more accurate prognostic stratification. Furthermore, the preoperative ADC obtained by MRI could be a clinical surrogate marker in the TME of HCC. This might be the first report to focus on both CAFs and TAMs in the TME of HCC and their correlation with the ADC.

The poor prognosis in CAF- or TAM-positive patients requires some discussion. Multiple

Table 1. Correlation between αSMA expression and clinicopathological variables

Factors	αSMA expression		*P*-value
	Negative (n=74)	Positive (n=35)	
Age: Mean ± SD	70±11	68±11	0.52
Gender			
Male/Female	57/17	23/12	0.21
BMI: Mean ± SD	24±3	23±3	0.21
Hepatic virus infection			
nBnC/HBV, HCV, HBCV	33/41	14/21	0.65
mALBI grade			
1, 2a/2b, 3	44/30	13/22	0.66
Tumor number			
Single/Multiple	61/13	21/14	0.01
Tumor size			
<5 cm/≥5 cm	42/32	21/14	0.74
Tumor differentiation			
Well, moderate/poor	65/9	31/4	0.91
Tumor growth type			
EG/IG	67/7	26/9	0.03
Portal invasion			
Absent/Present	62/12	27/8	0.40
Venous invasion			
Absent/Present	66/8	31/4	0.92
Stage			
I, II/III, IV	49/25	14/21	<0.01
BCLC			
0, A/B, C	61/13	25/10	0.19
AFP (ng/ml)			
<400/≥400	61/13	28/7	0.75
DCP (mAU/ml)			
<500/≥500	45/29	19/16	0.52

markers have been found to identify CAFs, including αSMA, FAP, fibroblast-specific protein 1, vimentin, and platelet-derived growth factor receptors-α and -β [30, 31]. In the present study, αSMA was selected as a CAF marker. CAF is a major component of the TME, providing physical support and secreting various proteins that can modulate tumor growth and survival, such as extracellular matrix proteins, hepatocyte growth factor, insulin-like growth factor 1/2, and cytokines [32]. We previously reported that interleukin-6 derived from CAFs promotes HCC progression through the JAK/STAT pathway [9]. Several studies have shown that CAFs have significant phenotypic and functional heterogeneity, and they can exhibit both protumorigenic and antitumorigenic activity [33]. Notably, CAFs modulate HCC progression not only via direct effects on HCC cells, but also via indirect effects through other cells in the TME. In this study, CAF expression was significantly correlated with TAM expression. Using a cytokine array analysis, we previously demonstrated that the interaction between CAFs and TAMs via the osteopontin pathway [11] and plasminogen activator inhibitor-1 pathway [12] increased tumor malignancy.

Macrophages can be classified as M1 and M2 according to their phenotype, and their markers comprise transmembrane glycoproteins, growth factors, hormones, cytokines, and cytokine receptors. TAMs are reportedly similar to M2 macrophages [34], expressing markers such as CD206, CD163, CD204, and arginase-1 [7, 8]. CD204 was selected as a TAM marker in the present study. Like CAFs, TAMs are also involved in HCC development, acquisition of tumor malignancy, and drug resistance [34]. We previously investigated the interaction between HCC and TAMs, focusing on NRF2 activation [7]. Macrophage NRF2 activation by cancer cell-derived lactate skews macrophage polarization toward an M2-like phenotype, and educated macrophages activate NRF2 in cancer cells to promote epithelial-mesenchymal transition through VEGF secretion from TAMs. The present study provides a new understanding of the role of NRF2 in cancer cells and TAM interactions and suggests a potential therapeutic target. Cancer cell-derived VEGF also activates TAMs [8].

The development of drugs that target CAFs and TAMs in HCC is urgently needed. In our previous study, we reported that TU-100 antagonized the activation of both CAFs [13] and TAMs [14] and that these cells could serve as therapeutic targets for HCC. VEGF is a crucial cytokine in the interaction between HCC and TAMs [7, 8]. Therefore, VEGF blockage by bevacizumab in

Table 2. Correlation between CD204 expression and clinicopathological variables

Factors	CD204 expression		*P*-value
	Negative (n=92)	Positive (n=17)	
Age: Mean ± SD	69±10	70±12	0.84
Gender			
Male/Female	67/25	13/4	0.75
BMI: Mean ± SD	24±3	23±4	0.81
Hepatic virus infection			
nBnC/HBV, HCV, HBCV	38/54	9/8	0.37
mALBI grade			
1, 2a/2b, 3	51/41	6/11	0.26
Tumor number			
Single/Multiple	72/20	10/7	0.09
Tumor size			
<5 cm/≥5 cm	53/39	10/7	0.93
Tumor differentiation			
Well, moderate/poor	81/11	15/2	0.98
Tumor growth type			
EG/IG	80/12	13/4	0.26
Portal invasion			
Absent/Present	75/17	14/3	0.94
Venous invasion			
Absent/Present	85/7	12/5	<0.01
Stage			
I, II/III, IV	55/37	8/9	0.33
BCLC			
0, A/B, C	73/19	13/4	0.79
AFP (ng/ml)			
<400/≥400	74/18	15/2	0.45
DCP (mAU/ml)			
<500/≥500	54/38	10/7	0.99

the TME could be an effective strategy for HCC. Although further examination is necessary, immune checkpoint inhibitors such as atezolizumab-bevacizumab [2] and durvalumab-tremelimumab [3] might regulate CAF and TAM activation. We suggest that CAF and TAM double-positive patients might be suitable for adjuvant chemotherapy after Hx.

Patients will benefit clinically if their TME status can be predicted using an examination that is less invasive than tumor biopsy. Therefore, we investigated whether several serum immune parameters that were candidate prognostic indicators for HCC in our previously published reports, including the total lymphocyte count, neutrophil-to-lymphocyte ratio [35], prognostic nutritional index [36], and lymphocyte to C-reactive protein ratio [37], were correlated with the expression of CAF/TAM markers. We found no significant association of these serum immune parameters and the expression of CAF/TAM markers (data not shown). Therefore, we focused on the ADC derived from MRI because it has been reported as a prognostic predictor in various cancers, including HCC [23-26]. Especially in IHCC, the ADC can reflect the status of microscopic tumor-infiltrating lymphocytes in tumor tissue [24]. Therefore, we investigated whether the ADC reflects CAF and TAM expression in the TME, and we found that the ADC was a clinical surrogate marker in the TME. In this study, CAF- and TAM-positive patients showed low tumoral ADCs. High numbers of intratumoral CAFs might promote tumor fibrosis, and highly dense tumors showed lower ADCs. Why the presence of numerous TAMs led to low ADCs remains unclear, but it is possible that some cytokines or exosomes from TAMs are related to highly dense tumors.

In the prognostic analysis, both CAF/TAM markers and the mALBI grade were independent prognostic factors for both OS and DFS. The mALBI grade has been shown to be a prognostic factor after chemotherapy [38] and to predict the incidence of postoperative liver failure after Hx [39]. In the present study, patients with better liver function were able to receive various additional treatments, including repeat Hx, when recurrence was detected. Therefore, the mALBI grade was also found to be a prognostic factor in the present study.

This study had three main limitations. First, this was a single-center retrospective cohort study with a relatively small number of patients. Although CD204 did not reach statistical significance for DFS, it showed significance for OS. Analysis of higher numbers of patients in future studies might reveal significant differences in DFS as well as OS. Second, only IHC was performed to investigate CAF and TAM expression.

Table 3. Univariate and multivariate analysis of OS

Variables	5-year survival rate	Univariate *p*-value	Hazard ratio	95% C.I.	Multivariate *p*-value
Age (yo) (<75/≥75)	79.8/74.0	0.53			
Gender (male/female)	78.3/78.0	0.91			
Virus (nBnC/HBV, HCV)	76.2/79.7	0.87			
mALBI grade (1, 2a/2b, 3)	88.7/68.3	<0.01	3.19	1.10-9.26	0.03
Tumor number (single/multiple)	86.8/55.5	<0.01	1.59	0.36-7.10	0.54
Tumor size (cm) (<5/>5)	83.1/71.6	0.12			
Differentiation (well, moderate/poor)	78.4/77.9	0.43			
Tumor growth pattern (EG/IG)	80.8/62.3	0.02	1.05	0.31-3.58	0.93
VP (-/+)	80.4/58.2	0.03	1.91	0.56-6.49	0.30
VV (-/+)	77.9/78.7	0.90			
Stage (I, II/III, IV)	86.7/62.6	<0.01	1.00	0.23-4.44	0.99
BCLC (0, A/B, C)	87.3/45.8	<0.01	2.96	0.81-16.13	0.21
AFP (ng/ml) (<400/>400)	79.9/72.1	0.19			
DCP (mAU/ml) (<500/>500)	87.3/65.6	0.04	2.24	0.81-6.17	0.12
αSMA/CD204 (other/double positive)	82.0/42.9	<0.01	3.27	1.20-8.93	0.02

Table 4. Univariate and multivariate analysis of DFS

Variables	5-year survival rate	Univariate *p*-value	Hazard ratio	95% C.I.	Multivariate *p*-value
Age (yo) (<75/≥75)	42.8/52.1	0.40			
Gender (male/female)	39.4/64.1	0.12			
Virus (nBnC/HBV, HCV)	40.6/50.1	0.90			
mALBI grade (1, 2a/2b, 3)	64.1/27.7	<0.01	2.79	1.51-5.18	<0.01
Tumor number (single/multiple)	54.9/20.8	<0.01	1.76	0.76-4.05	0.18
Tumor size (cm) (<5/>5)	49.8/43.5	0.11			
Differentiation (well, moderate/poor)	47.4/36.4	0.62			
Tumor growth pattern (EG/IG)	51.1/8.6	<0.01	1.57	0.71-3.50	0.27
VP (-/+)	48.3/37.6	0.20			
VV (-/+)	49.1/NA	0.08			
Stage (I, II/III, IV)	58.8/29.3	<0.01	1.81	0.89-3.70	0.10
BCLC (0, A/B, C)	53.2/10.1	<0.01	0.96	0.34-2.75	0.94
AFP (ng/ml) (<400/>400)	44.4/54.8	0.37			
DCP (mAU/ml) (<500/>500)	51.8/32.8	0.08			
αSMA/CD204 (other/double positive)	50.1/0.0	0.01	1.34	0.73-2.46	0.35

However, because various in vitro studies had already been published by our department, the true purpose of this study was to analyze clinical samples. Third, not only tissue samples but also liquid samples should be collected both before and after surgery. Therefore, we have recently begun collecting liquid samples from all patients undergoing surgical treatment of HCC.

In conclusion, both CAF and TAM marker expression reflected a poor prognosis after Hx. It is critical to analyze not only single markers but also the combination of CAF and TAM markers for more accurate prognostic stratification. Furthermore, the preoperative ADC may serve as a clinical surrogate marker in the TME of HCC.

Conclusions

In conclusion, both CAF and TAM marker expression reflected poor prognosis after Hx. It was critical to analyze not only a single marker

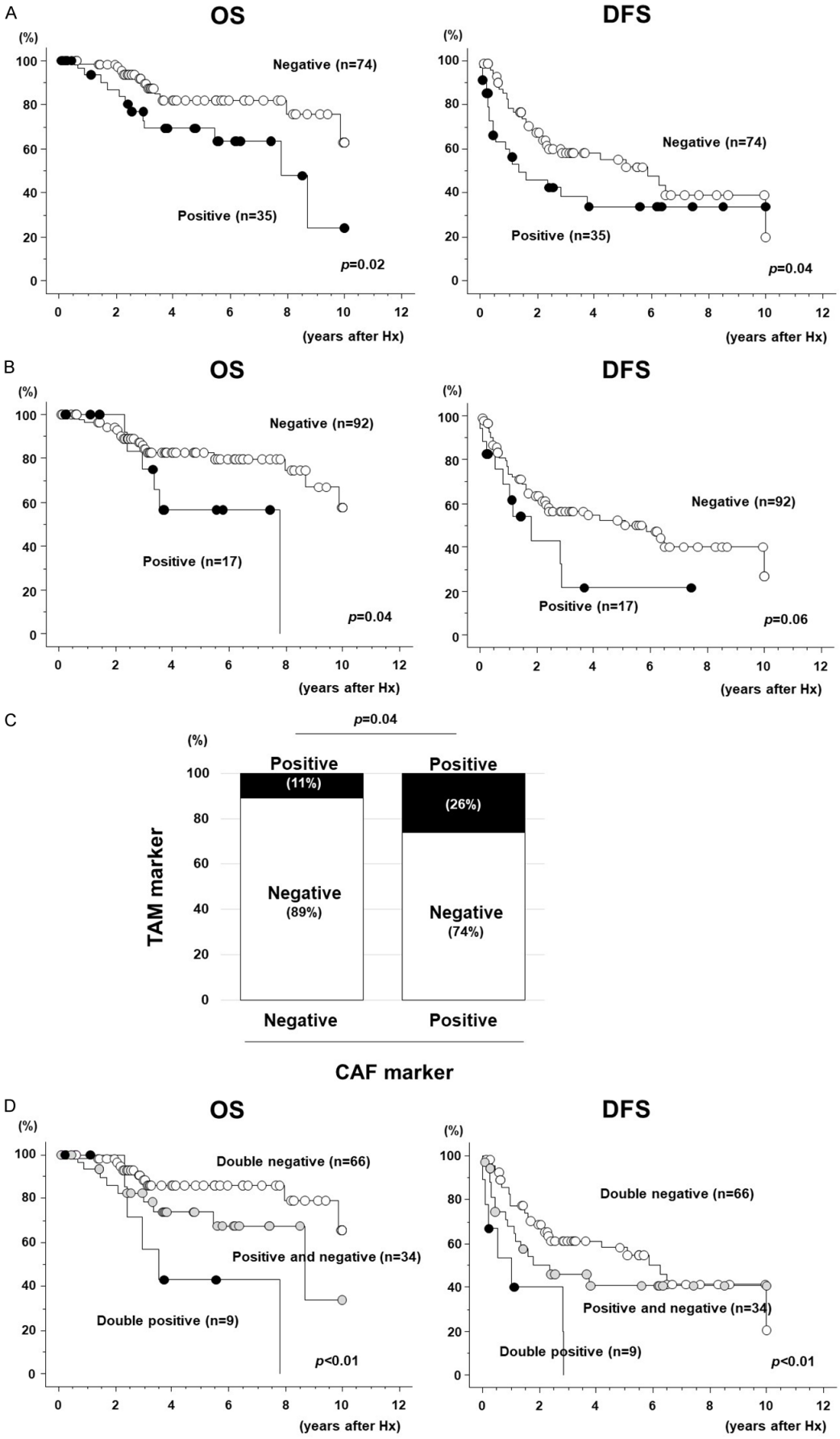
A
OS
(%)
Negative (n=74)
Positive (n=35)
p=0.02
(years after Hx)
DFS
(%)
Negative (n=74)
Positive (n=35)
p=0.04
(years after Hx)
B
OS
(%)
Negative (n=92)
Positive (n=17)
p=0.04
(years after Hx)
DFS
(%)
Negative (n=92)
Positive (n=17)
p=0.06
(years after Hx)
C
p=0.04
(%)
TAM marker
Positive
(11%)
Negative
(89%)
Positive
(26%)
Negative
(74%)
Negative
Positive
CAF marker
D
OS
(%)
Double negative (n=66)
Positive and negative (n=34)
Double positive (n=9)
p<0.01
(years after Hx)
DFS
(%)
Double negative (n=66)
Positive and negative (n=34)
Double positive (n=9)
p<0.01
(years after Hx)

Figure 4. Long-term prognosis. A. CAF marker. CAF marker positive patients showed poor prognosis after Hx in both OS (*P*=0.02) and DFS (*P*=0.04). B. TAM marker. TAM marker positive patients showed poor prognosis after Hx in both OS (*P*=0.04) and DFS (*P*=0.06). C. Correlation between CAF and TAM expression. The expression of CAF and TAM marker had positive correlation (*P*=0.04). D. Prognostic stratification with both CAF and TAM markers. When patients were divided into a CAF/TAM double-negative group, a CAF-positive or TAM-positive group, and a CAF/TAM double-negative group, both OS and DFS were clearly stratified by these two markers (P<0.01).

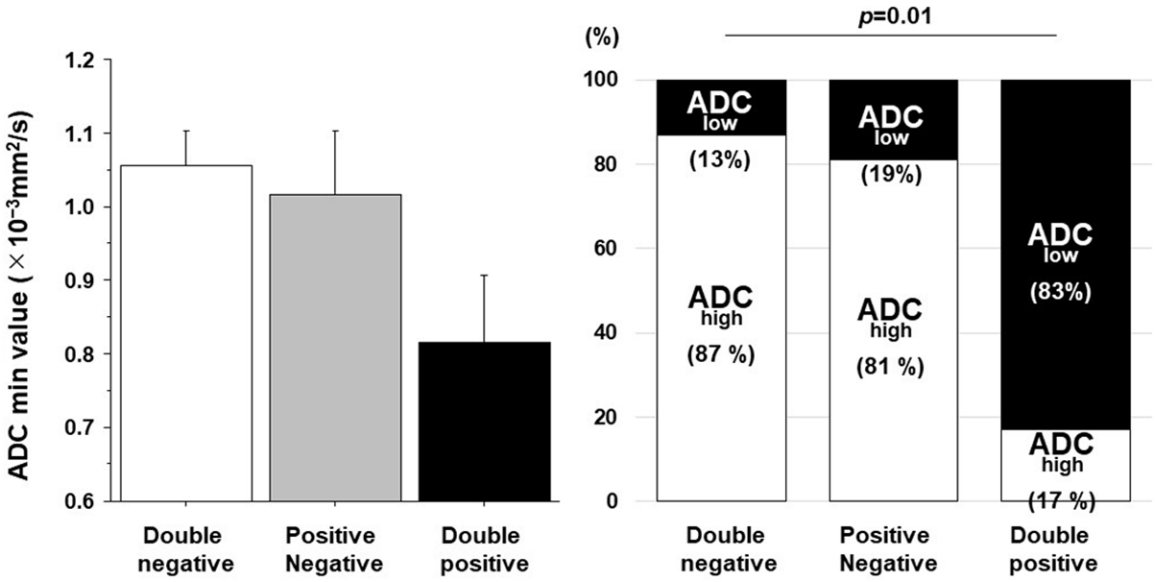

Figure 5. ADC value as clinical surrogate marker in TME. ADC minimum values of CAF/TAM double positive group showed lower value than other groups (Double negative group; 1.06±0.05, Positive and negative group; 1.02±0.09, Double positive group; 0.82±0.09, *P*=0.06; Double negative vs. Double positive group). Under the criteria of cut-off value; 0.74×10^{-3} mm^2/s, CAF/TAM double positive group showed significantly higher ADC^{low} rate (Double negative group; 13%, Positive and negative group; 19%, Double positive group; 83%, *P*=0.01).

but also double CAF and TAM markers for more accurate prognostic stratification. Furthermore, preoperative ADC value could be clinical surrogate marker in TME in HCC patients.

Disclosure of conflict of interest

None.

Abbreviations

CAF, cancer-associated fibroblast; TAM, tumor-associated macrophage; HCC, hepatocellular carcinoma; Hx, hepatectomy; ADC, apparent diffusion coefficient; DWI, diffusion-weighted image; αSMA, alpha smooth muscle actin; ICI, immune checkpoint inhibitor; PD-1, programmed death receptor-1; PD-L1, programmed death ligand 1; CTLA-4, cytotoxic T-lymphocyte antigen-4; TME, tumor microenvironment; NK, natural killer; MDSC, myeloid derived suppressor cells; Nrf2, nuclear factor (erythroid-derived 2)-like 2; EMT, epithelial-mesenchymal transition; JAK/STAT, Janus kinase/signal transducer and activator of transcription; BAFF, B-cell activating factor; FAP, fibroblast activation protein; AFP, alpha fetoprotein; DCP, des-gamma-carboxy prothrombin; CT, computed tomography; Gd-EOB-DTPA, gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid; MRI, magnetic resonance imaging; IHC, immunohistochemistry; HPF, high-powered field; OS, overall survival; DFS, disease-free survival; FAP, fibroblast activation protein; FSP1, fibroblast-specific protein 1; PDGFRs, vimentin and PDGF receptor; ECMs, extracellular matrices; HGF, hepatocyte growth factor; ILGF1/2, insulin-like growth factor 1/2.

Address correspondence to: Dr. Yu Saito, Department of Surgery, Tokushima University, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan. Tel: +81-88-633-9276; Fax: +81-88-631-9698; E-mail: saito.yu.1001@tokushima-u.ac.jp

References

[1] Rimassa L, Finn RS and Sangro B. Combination immunotherapy for hepatocellular carcinoma. J Hepatol 2023; 79: 506-15.

[2] Finn RS, Qin S, Ikeda M, Galle PR, Ducreux M, Kim TY, Kudo M, Breder V, Merle P, Kaseb AO, Li D, Verret W, Xu DZ, Hernandez S, Liu J, Huang C, Mulla S, Wang Y, Lim HY, Zhu AX and Cheng AL; IMbrave150 Investigators. Atezolizumab plus bevacizumab in unresectable hepatocellular carcinoma. N Engl J Med 2020; 382: 1894-905.

[3] Abou-Alfa GK, Lau G, Kudo M, Chan SL, Kelley RK, Furuse J, Sukeepaisarnjaroen W, Kang YK, Van Dao T, De Toni EN, Rimassa L, Breder V, Vasilyev A, Heurgué A, Tam VC, Mody K, Thungappa SC, Ostapenko Y, Yau T, Azevedo S, Varela M, Cheng AL, Qin S, Galle PR, Ali S, Marcovitz M, Makowsky M, He P, Kurland JF, Negro A and Sangro B. Tremelimumab plus durvalumab in unresectable hepatocellular carcinoma. NEJM Evid 2022; 1: EVIDoa2100070.

[4] Balkwill F and Mantovani A. Inflammation and cancer: back to Virchow? Lancet 2001; 357: 539-45.

[5] Medzhitov R. Origin and physiological roles of inflammation. Nature 2008; 454: 428-35.

[6] Gajewski TF, Schreiber H and Fu YX. Innate and adaptive immune cells in the tumor microenvironment. Nat Immunol 2013; 14: 1014-22.

[7] Feng R, Morine Y, Ikemoto T, Imura S, Iwahashi S, Saito Y and Shimada M. Nrf2 activation drive macrophages polarization and cancer cell epithelial-mesenchymal transition during interaction. Cell Commun Signal 2018; 16: 54.

[8] Okikawa S, Morine Y, Saito Y, Yamada S, Tokuda K, Teraoku H, Miyazaki K, Yamashita S, Ikemoto T, Imura S and Shimada M. Inhibition of the VEGF signaling pathway attenuates tumor-associated macrophage activity in liver cancer. Oncol Rep 2022; 47: 71.

[9] Iwahasi S, Rui F, Morine Y, Yamada S, Saito YU, Ikemoto T, Imura S and Shimada M. Hepatic stellate cells contribute to the tumor malignancy of hepatocellular carcinoma through the IL-6 pathway. Anticancer Res 2020; 40: 743-9.

[10] Gao L, Morine Y, Yamada S, Saito Y, Ikemoto T, Tokuda K, Miyazaki K, Okikawa S, Takasu C and Shimada M. The BAFF/NFkappaB axis is crucial to interactions between sorafenib-resistant HCC cells and cancer-associated fibroblasts. Cancer Sci 2021; 112: 3545-54.

[11] Tokuda K, Morine Y, Miyazaki K, Yamada S, Saito Y, Nishi M, Tokunaga T, Ikemoto T, Imura S and Shimada M. The interaction between cancer associated fibroblasts and tumor associated macrophages via the osteopontin pathway in the tumor microenvironment of hepatocellular carcinoma. Oncotarget 2021; 12: 333-43.

[12] Chen S, Morine Y, Tokuda K, Yamada S, Saito Y, Nishi M, Ikemoto T and Shimada M. Cancer-associated fibroblast-induced M2-polarized macrophages promote hepatocellular carcinoma progression via the plasminogen activator inhibitor-1 pathway. Int J Oncol 2021; 59: 59.

[13] Wada Y, Tokuda K, Morine Y, Okikawa S, Yamashita S, Ikemoto T, Imura S, Saito Y, Yamada S and Shimada M. The inhibitory effect of TU-100 on hepatic stellate cell activation in the tumor microenvironment. Oncotarget 2020; 11: 4593-604.

[14] Chen S, Morine Y, Saito YU, Yamada S, Teraoku H, Ikemoto T and Shimada M. TU-100 antagonizes the M2 polarization phenotype of macrophages in the tumor microenvironment by suppressing the TLR4/NF-B/STAT3 axis. Anticancer Res 2023; 43: 1985-92.

[15] Zou B, Liu X, Zhang B, Gong Y, Cai C, Li P, Chen J, Xing S, Chen J, Peng S, Pokhrel B, Ding L, Zeng L and Li J. The expression of FAP in hepatocellular carcinoma cells is induced by hypoxia and correlates with poor clinical outcomes. J Cancer 2018; 9: 3278-86.

[16] Sun B, Lei X, Cao M, Li Y and Yang LY. Hepatocellular carcinoma cells remodel the pro-metastatic tumour microenvironment through recruitment and activation of fibroblasts via paracrine Egfl7 signaling. Cell Commun Signal 2023; 21: 180.

[17] Kono H, Fujii H, Furuya S, Hara M, Hirayama K, Akazawa Y, Nakata Y, Tsuchiya M, Hosomura N and Sun C. Macrophage colony-stimulating factor expressed in non-cancer tissues provides predictive powers for recurrence in hepatocellular carcinoma. World J Gastroenterol 2016; 22: 8779-89.

[18] Yusa T, Yamashita YI, Okabe H, Nakao Y, Itoyama R, Kitano Y, Kaida T, Miyata T, Mima K, Imai K, Hayashi H and Baba H. Survival impact of immune cells infiltrating peritumoral area of hepatocellular carcinoma. Cancer Sci 2022; 113: 4048-58.

[19] Liao J, Zeng DN, Li JZ, Hua QM, Huang CX, Xu J, Wu C, Zheng L, Wen WP and Wu Y. Type I IFNs repolarized a CD169(+) macrophage population with anti-tumor potentials in hepatocellular carcinoma. Mol Ther 2022; 30: 632-43.

[20] Li JQ, Yu XJ, Wang YC, Huang LY, Liu CQ, Zheng L, Fang YJ and Xu J. Distinct patterns and prognostic values of tumor-infiltrating macrophages in hepatocellular carcinoma and gastric cancer. J Transl Med 2017; 15: 37.

[21] Jiang T, Xu JH, Zou Y, Chen R, Peng LR, Zhou ZD and Yang M. Diffusion-weighted imaging (DWI) of hepatocellular carcinomas: a retrospective analysis of the correlation between qualitative

and quantitative DWI and tumour grade. Clin Radiol 2017; 72: 465-72.
[22] Kurosawa J, Tawada K, Mikata R, Ishihara T, Tsuyuguchi T, Saito M, Shimofusa R, Yoshitomi H, Ohtsuka M, Miyazaki M and Yokosuka O. Prognostic relevance of apparent diffusion coefficient obtained by diffusion-weighted MRI in pancreatic cancer. J Magn Reson Imaging 2015; 42: 1532-7.
[23] Yamada S, Morine Y, Imura S, Ikemoto T, Arakawa Y, Saito Y, Yoshikawa M, Miyazaki K and Shimada M. Prognostic prediction of apparent diffusion coefficient obtained by diffusion-weighted MRI in mass-forming intrahepatic cholangiocarcinoma. J Hepatobiliary Pancreat Sci 2020; 27: 388-95.
[24] Miyazaki K, Morine Y, Yamada S, Saito Y, Tokuda K, Okikawa S, Yamashita S, Oya T, Ikemoto T, Imura S, Hu H, Morioka H, Tsuneyama K and Shimada M. Stromal tumor-infiltrating lymphocytes level as a prognostic factor for resected intrahepatic cholangiocarcinoma and its prediction by apparent diffusion coefficient. Int J Clin Oncol 2021; 26: 2265-74.
[25] Yoshikawa M, Morine Y, Yamada S, Miyazaki K, Tokuda K, Saito Y, Arakawa Y, Ikemoto T, Imura S and Shimada M. Prognostic prediction of resectable colorectal liver metastasis using the apparent diffusion coefficient from diffusion-weighted magnetic resonance imaging. Ann Gastroenterol Surg 2020; 5: 252-8.
[26] Yamada S, Morine Y, Ikemoto T, Saito Y, Teraoku H, Waki Y, Nakasu C and Shimada M. Impact of apparent diffusion coefficient on prognosis of early hepatocellular carcinoma: a case control study. BMC Surg 2023; 23: 6.
[27] Selenge B, Yamada S, Morine Y, Ikemoto T, Saito Y, Takasu C, Teraoku H and Shimada M. Impact of L-type amino acid transporter 3 on the prognosis of hepatocellular carcinoma. Oncol Lett 2022; 24: 418.
[28] Meng J, Zhou Y, Lu X, Bian Z, Chen Y, Zhou J, Zhang L, Hao Z, Zhang M and Liang C. Immune response drives outcomes in prostate cancer: implications for immunotherapy. Mol Oncol 2021; 15: 1358-75.
[29] Miyasato Y, Shiota T, Ohnishi K, Pan C, Yano H, Horlad H, Yamamoto Y, Yamamoto-Ibusuki M, Iwase H, Takeya M and Komohara Y. High density of CD204-positive macrophages predicts worse clinical prognosis in patients with breast cancer. Cancer Sci 2017; 108: 1693-700.
[30] Akkiz H. Emerging role of cancer-associated fibroblasts in progression and treatment of hepatocellular carcinoma. Int J Mol Sci 2023; 24: 3941.
[31] Peng H, Zhu E and Zhang Y. Advances of cancer-associated fibroblasts in liver cancer. Biomark Res 2022; 10: 59.
[32] Xu H, Zhao J, Li J, Zhu Z, Cui Z, Liu R, Lu R, Yao Z and Xu Q. Cancer associated fibroblast-derived CCL5 promotes hepatocellular carcinoma metastasis through activating HIF1alpha/ZEB1 axis. Cell Death Dis 2022; 13: 478.
[33] Binnewies M, Roberts EW, Kersten K, Chan V, Fearon DF, Merad M, Coussens LM, Gabrilovich DI, Ostrand-Rosenberg S, Hedrick CC, Vonderheide RH, Pittet MJ, Jain RK, Zou W, Howcroft TK, Woodhouse EC, Weinberg RA and Krummel MF. Understanding the tumor immune microenvironment (TIME) for effective therapy. Nat Med 2018; 24: 541-50.
[34] Zhang X, Yu C, Zhao S, Wang M, Shang L, Zhou J and Ma Y. The role of tumor-associated macrophages in hepatocellular carcinoma progression: a narrative review. Cancer Med 2023; 12: 22109-29.
[35] Saito Y, Morine Y, Ikemoto T, Yamada S, Teraoku H, Yasui-Yamada S, Nishi M, Sakaue H, Kamada M, Matsuura T and Shimada M. Preoperative weight loss program for hepatocellular carcinoma patients with high body mass index in hepatectomy. World J Surg 2023; 47: 3348-55.
[36] Saito Y, Imura S, Morine Y, Ikemoto T, Yamada S and Shimada M. Preoperative prognostic nutritional index predicts short- and long-term outcomes after liver resection in patients with hepatocellular carcinoma. Oncol Lett 2021; 21: 153.
[37] Miyazaki K, Morine Y, Imura S, Ikemoto T, Saito Y, Yamada S, Tokuda K, Okikawa S, Yamashita S, Oya T, Tsuneyama K and Shimada M. Preoperative lymphocyte/C-reactive protein ratio and its correlation with CD8(+) tumor-infiltrating lymphocytes as a predictor of prognosis after resection of intrahepatic cholangiocarcinoma. Surg Today 2021; 51: 1985-95.
[38] Hsu WF, Hsu SC, Chen TH, Lin CH, Lin YC, Chang YW, Wang HW, Liao YM, Lai HC and Peng CY. Modified albumin-bilirubin model for stratifying survival in patients with hepatocellular carcinoma receiving anticancer therapy. Cancers (Basel) 2022; 14: 5083.
[39] Xu MH, Xu B, Zhou CH, Xue Z, Chen ZS, Xu WX, Huang C, Zhu XD, Zhou J, Fan J, Sun HC and Shen YH. An mALBI-Child-Pugh-based nomogram for predicting post-hepatectomy liver failure grade B-C in patients with huge hepatocellular carcinoma: a multi-institutional study. World J Surg Oncol 2022; 20: 206.

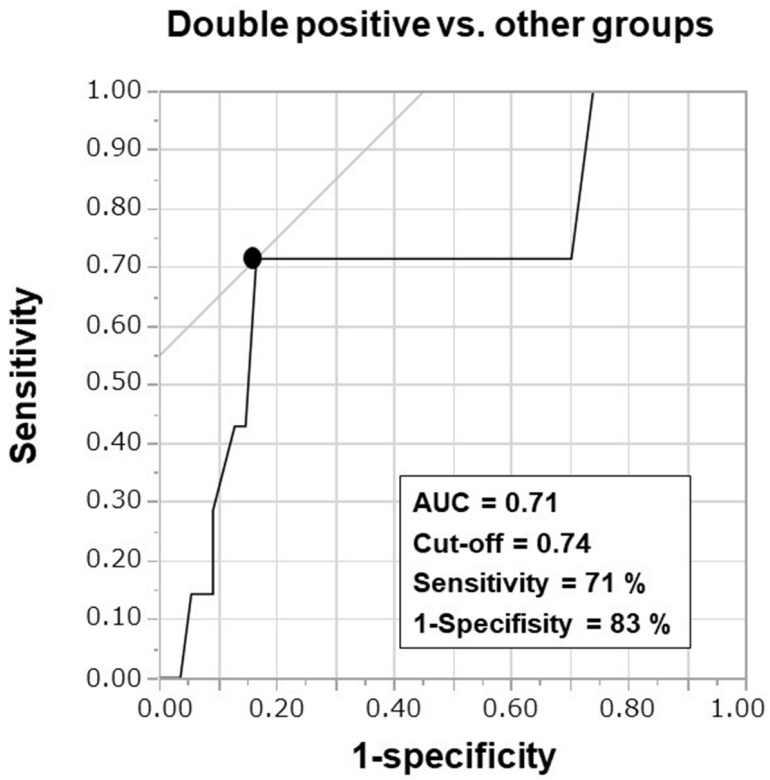

Figure S1. ROC for predicting CAF/TAM double positive patients. The cutoff value of minimum ADC value was 0.74×10^{-3} mm^2/s, calculated by a receiver operating characteristic curve for predicting whether CAF/TAM double positive patients or not. Sensitivity was calculated as 71%, and 1 - Specificity as 83% respectively.