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ORIGINAL ARTICLE

Expression of carbonic anhydrase 9 is a novel prognostic marker in resectable hepatocellular carcinoma

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Abstract Carbonic anhydrase 9 (CA9), which regulates cellular proliferation and the acid-base balance, is known as prognostic factor in various types of cancer. The aim of this study is to investigate the clinical implications of CA9 expression in patients with hepatocellular carcinoma. Immunohistochemical staining for CA9 was performed on tissue microarrays of hepatocellular carcinoma and paired non-neoplastic liver tissue from a training cohort of 838 patients and a validation cohort of 225 patients. Membranous staining in more than 5 % of the tumor cells was considered to indicate CA9 positivity. The prognostic value of CA9 expression was statistically evaluated. In the training cohort, CA9 positivity (181 cases, 21.5 %) was significantly correlated with shorter overall survival (OS; $p<0.001$) and recurrence-free survival (RFS; $p=0.004$). In multivariate analysis, CA9 positivity was independently associated with reduced OS ($p=0.023$), but not significantly associated with RFS ($p=0.384$). These results were validated in an additional cohort (CA9 positivity in 35 cases, 15.6 %; OS, $p=0.015$; RFS, $p=0.979$). Pooled cohort analysis showed that this predictor was independently associated with higher mortality (OS; $p<0.001$). These data indicate that CA9 expression is a poor prognostic factor in resectable hepatocellular carcinoma (HCC) patients.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, and its incidence is increasing because of the dissemination of hepatitis B and C virus [1, 2]. It is also the third most common cause of cancer-related deaths, with more than 20,000 estimated deaths in the USA in 2013 [3, 4]. Most patients with HCC already have advanced stage disease at the time of diagnosis, and approximately 80 % of patients with advanced HCC cannot undergo curative surgical resection [5]. The median survival in unresectable patients with HCC is less than 4 months [6]. Even after surgical resection, the 5-year survival rate is approximately 25 to 40 %, and the 2-year tumor recurrence rate is as high as 55 % [4, 6].

Pathological and clinical factors, including venous invasion, the presence of satellite nodules, and multiple tumors as well as a large tumor size, are prognostic for survival and recurrence after surgical resection of patients with HCC [7]. In recent studies, tumor biologic factors, such as proliferative and angiogenic activity, have been reported as new prognostic factors for HCC [7–9]. Because postoperative recurrence and mortality are not fully predicted using the above-mentioned factors, the discovery of new predictive or prognostic factors would help to identify when additional treatment is required and might improve survival after surgical resection.

Hypoxia of tumor cells produces intracellular acidification that activates genes involved in the adaptation to hypoxic stress [10]. Carbonic anhydrase 9 (CA9), which is one of the genes most strongly induced by hypoxia, regulates cell proliferation and the acid-base balance [11]. CA9 is expressed in more than 85 % of clear cell renal cell carcinomas, and it is thus used as a diagnostic marker [12]. More recent studies

revealed that CA9 is expressed in a wide variety of malignancies, including those of the lung, breast, colorectum, bladder, cervix, and head and neck [13–20]. CA9 expression in tumor cells is related to poor prognosis and may be used as a marker of an aggressive malignant behavior, predictive of progression [21]. Although some studies recently reported an association between hypoxia and CA9 expression in HCC cells in vitro, the significance of CA9 expression in surgically resected HCC is unknown [10, 22].

In this study, we performed immunohistochemical staining for CA9 on tissue microarrays of HCC. We analyzed the staining results in conjunction with clinicopathological variables to investigate the clinical significance of CA9 expression and to determine the therapeutic potential of a CA9 inhibitor in HCC patients.

Materials and methods

Patients and samples

This retrospective study included 1063 patients (training cohort, 838 patients; validation cohort, 225 patients) diagnosed with HCC at Asan Medical Center between 1999 and 2011 by surgical resection. All clinical information was collected from the database of the Asan Liver Center, Seoul, Korea. The training cohort consisted of 838 cases, selected from all 883 surgically resected HCC cases between 1999 and 2004 by excluding 13 cases without paraffin blocks, 18 cases of combined HCC and cholangiocarcinoma, 10 recurrent cases from patients who were already in the cohort, 2 cases without clinical information, and 2 cases of which the tissue cores were lost from the TMA during immunostaining (Fig. 1). Of the 838 patients, 180 (21.5 %) underwent liver transplantation for tumor control and 658 (78.5 %) hepatectomy without a previous operation for HCC. Furthermore, 312 patients (37.2 %) had been treated for HCC [portal vein embolization (PVE), 36 patients; transcatheter arterial chemoembolization (TACE), 236 patients; radiofrequency ablation (RFA), 4 patients; TACE+PVE, 28 patients; TACE+RFA, 8 patients]. For the validation study, 225 patients with HCC were selected from 2586 patients who underwent hepatectomy between 2005 and 2011. Only two patients previously underwent PVE. All specimens were enrolled and stored after operation in the Bio-Resource Center of Asan Medical Center, Seoul, Korea. No patients had Child-Pugh class B or C and portal vein (PV) or hepatic vein (HV) invasion.

Clinical assessment and follow-up

Clinical information, including age, sex, serum alpha-fetoprotein (AFP) level, hepatitis virus, Child-Pugh class, and survival data, was obtained by reviewing the medical records. All available slides were reviewed without knowledge of the clinical information, and pathological characteristics were collected. The median follow-up time was 84 months in the training cohorts (range, 2 days–176 months) and 57 months in the validation cohort (range, 5–104 months). For evaluation of recurrence after surgery, routine follow-up imaging analysis with liver protocol dynamic computed tomography scans and laboratory tests including serum AFP assays were performed 1 month after resection, every 3 months for the first 2 years, and every 3 to 6 months thereafter. Ninety-five patients of the training cohort were lost to follow-up and excluded from the assessment of recurrence-free survival (RFS). No patients were lost to follow-up in the validation cohort.

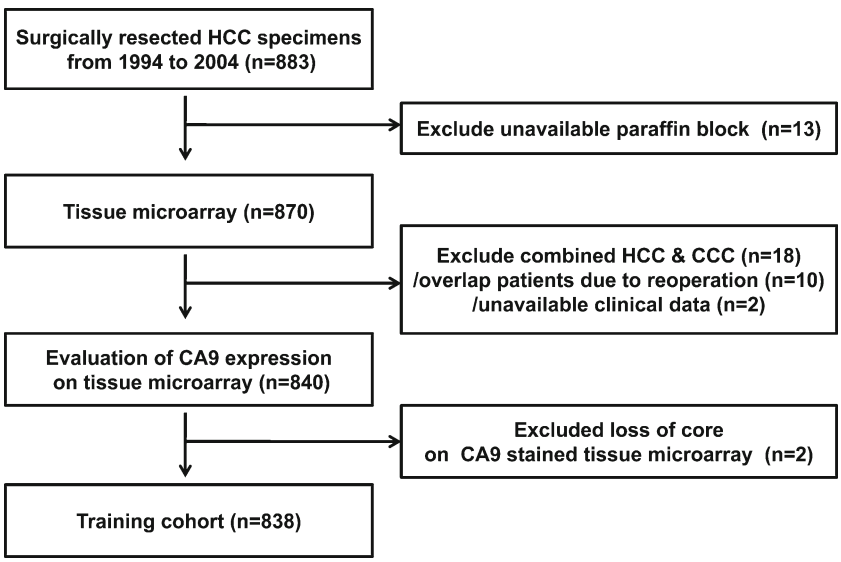

Fig. 1 Flow diagram showing exclusion criteria for the selection of hepatocellular carcinoma patients

Table 1 Results of CA9 immunostaining in hepatocellular carcinoma

Score	CA9 expression in hepatocellular carcinoma	Results
0	No expression	Negative
1	<5 %,weakly membranous staining	Negative
2	5 -<25 %, moderately to strongly membranous staining	Low positive
3	25 -<50 %, moderately to strongly membranous staining	Low positive
4	50 -<75 %, moderately to strongly membranous staining	High positive
5	≥75 %, moderately to strongly membranous staining	High positive

Tissue microarrays

A total of 68 tissue microarrays (870 cases in 55 tissue microarrays of training cohort and 227 cases in 13 tissue microarrays of validation cohort) were constructed from formalin-fixed, paraffin-embedded tissue samples from surgically resected HCC and adjacent non-neoplastic liver tissue. Hematoxylin and eosin (H&E)-stained slides were examined by two pathologists (EY, HJK) to identify tumor and normal tissues. Representative areas of tumor and adjacent non-neoplastic liver tissue were marked on H&E-stained slides using different color pens. Two 1.5-mm cores from the tumor and one core from the adjacent non-neoplastic liver tissue were arrayed from the corresponding paraffin blocks into a recipient block with holes created using an arraying machine (TMArrayer; Pathology Devices, Westminster, MD).

Immunohistochemical staining and evaluation

All tissue microarray blocks were immunohistochemically stained for CA9 (NB 100–417, rabbit polyclonal, 1:1000 dilution; Novus, Littleton, CO) using a Bench Mark XT automatic immunostaining device (Ventana Medical System, Tucson, AZ) with an OptiView DAB IHC Detection Kit (Ventana Medical System) according to the manufacturers' instructions. Tissue sections (4 μm) were transferred onto silanized slides and allowed to dry (10 min at room temperature, followed by 20 min in an incubator at 65 °C), subjected to heat-induced epitope retrieval using Cell Conditioning 1 (CC1) buffer for 32 min and incubated for 16 min with CA9 antibody in the autoimmunostainer.

Evaluation of immunostaining was independently performed by two pathologists (EY, HJK). The agreement between the two pathologists was more than 95 %. Discordant cases were resolved by review with a hepatobiliary pathologist. As in the positive control (clear cell renal cell carcinoma), CA9 was expressed along the cell membrane of HCC cells.

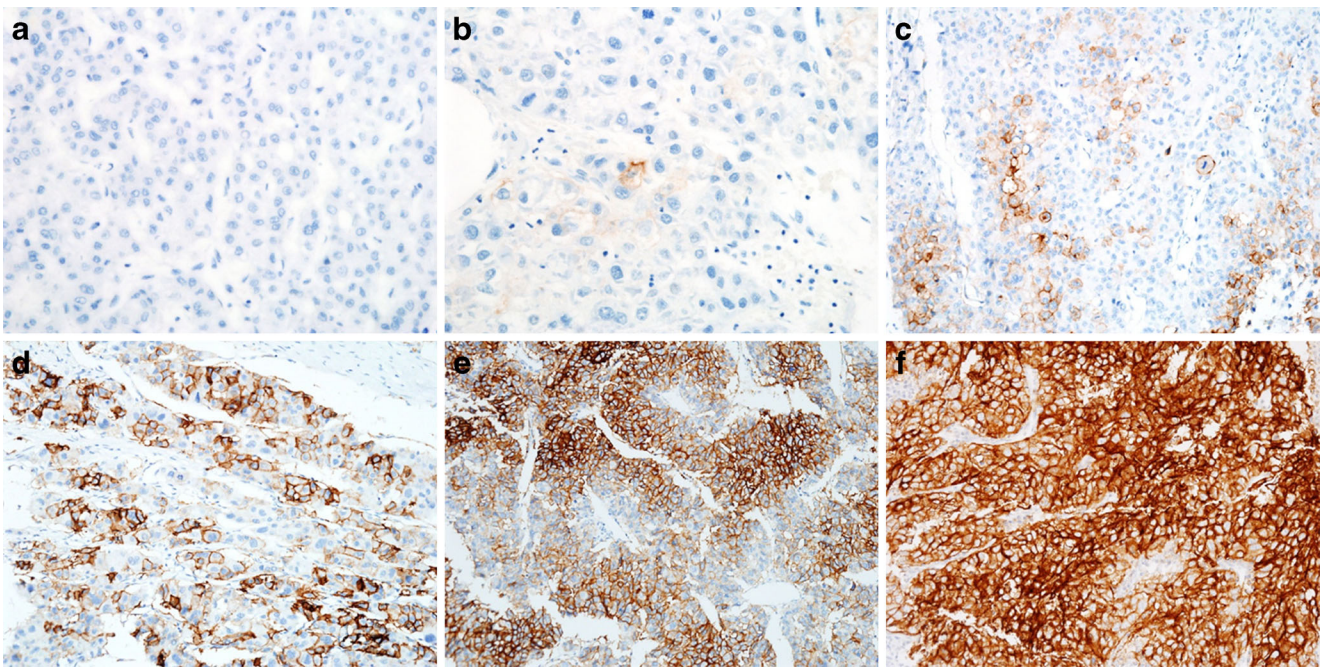

Fig. 2 CA9 expression in hepatocellular carcinoma. **a** *0* no expression (original magnification, ×400). **b** *1+* less than 5 % of cells showing weak membranous expression (original magnification, ×400). **c** *2+* between 5 and 25 % of cells showing moderate to strong membranous expression (original magnification, ×200). **d** *3+* between 25 and 50 % of cells showing moderate to strong membranous expression (original magnification ×200). **e** *4+* between 50 and 75 % of cells showing moderate to strong membranous expression (original magnification, ×200). **f** *5+* more than 75 % of cells showing moderate to strong membranous expression (original magnification, ×200)

Table 2 Demographic and clinical characteristics of patients

	Training cohort (%) (n=838)	Validation cohort (%) (n=225)
Mean age (range), years	51 (17~81)	55 (26~80)
Sex		
Male	684 (81.6)	169 (75.1)
Female	154 (18.4)	56 (24.9)
Mean serum AFP[a]		
≤100 ng/ml	436 (52.5)	131 (58.2)
>100 ng/ml	394 (47.5)	94 (41.8)
Hepatitis		
Hepatitis B	730 (87.1)	160 (71.1)
Hepatitis C	48 (5.7)	21 (9.3)
Hepatitis B+C	16 (1.9)	3 (1.3)
NBNC	44 (5.3)	41 (18.3)
Liver cirrhosis		
Absent	276 (32.9)	117 (52.0)
Present	562 (67.1)	108 (48.0)
CP class A	394 (70.1)	108 (100)
CP class B/C	168 (29.9)	0 (0)
Mean tumor size (range), cm	5.52 (1~21)	4.64 (1.2~18.0)
≤5 cm	532 (63.5)	166 (74.0)
>5 cm	306 (36.5)	59 (26.0)
Multiplicity		
Solitary	721 (86.0)	207 (92.0)
Multiple	117 (14.0)	18 (8.0)
Edmondson-Steiner grade		
I or II	275 (32.8)	146 (64.9)
III or IV	563 (67.2)	79 (35.1)
Microvascular invasion		
Absent	521 (62.2)	183 (81.3)
Present	317 (37.8)	42 (18.7)
PV or HV invasion[b]		
Absent	743 (89.7)	225 (100)
Present	85 (10.3)	0 (0)
Glisson capsule invasion		
Absent	734 (87.6)	214 (95.1)
Present	104 (12.4)	11 (4.9)
Previous treatment		
Absent	526 (62.8)	223 (99.1)
PVE	36 (4.3)	2 (0.9)
TACE	236 (28.2)	0 (0)
RFA	4 (0.5)	0 (0)
TACE+PVE	28 (3.3)	0 (0)
TACE+RFA	8 (0.9)	0 (0)

AFP alpha-fetoprotein, *NBNC* neither hepatitis B virus nor hepatitis C virus, *CP class* Child-Pugh class, *PV* portal vein, *HV* hepatic vein, *PVE* portal vein embolization, *TACE* transcatheter arterial chemoembolization, *RFA* radiofrequency ablation

[a] Only 830 patients with available information on serum AFP in the training cohort

[b] Only 828 patients with available information on PV or HV invasion in the training cohort

The expression of CA9 was scored according to the proportion of cells stained and staining intensity (Table 1). Cases containing more than 5 % tumor cells with moderate to strong membranous expression were considered positive for CA9 in HCC. Cases were clustered into three groups: a CA9-positive group subdivided into a low CA9-positive group (scores 2 and 3) and a high CA9-positive group (scores 4 and 5) and a CA9-negative group (scores 0 and 1) (Fig. 2).

Statistical analysis

Statistical analyses were performed using the R program (version 2.15.1: http://www.r-project.org). The χ^2 test was used to compare frequencies of categorical variables between groups. Overall survival (OS) was based on the time from the day of operation until death from any cause. The follow-up of patients still alive was censored at the last date of follow-up. RFS was assessed from the day of operation until relapse or the last follow-up date. OS and RFS were calculated using the Kaplan-Meier method, and statistical significances were evaluated using the log-rank test and the Cox proportional hazards regression model. All tests were two-sided, and p values less than 0.05 were considered statistically significant.

Results

Clinicopathological characteristics

The characteristics of the 838 patients included in the training cohort are listed in Table 2. The mean age at the time of diagnosis was 51 years (range, 17–81 years), and 684 patients (81.6 %) were male. Relapse occurred in 442 patients (52.7 %). The number of deaths due to HCC-related causes was 393 (46.9 %), and 78 patients (9.3 %) died of other causes. Median OS was 98, and median RFS was 49 months. The estimated 5-year OS and RFS rates were 59.4 and 48.0 %, respectively.

Correlation of CA9 expression with clinicopathological variables

In the training cohort, 181 HCC (21.6 %) had more than 5 % cells with moderate to strong membranous expression of CA9 (Fig. 3). CA9 was not expressed in non-neoplastic liver tissue. The correlations between CA9 expression and clinicopathological variables are summarized in Table 2. CA9 positivity significantly correlated with a high level of serum AFP ($p<0.001$), a tumor size larger than 5 cm ($p<0.001$), high Edmondson-Steiner grade ($p<0.001$), microvascular invasion ($p<0.001$), PV or HV invasion ($p<0.001$), and Glisson capsule invasion ($p=0.002$). CA9 expression was not significantly different between preoperatively treated and treatment naïve HCCs ($p>0.999$).

Prognostic significance of CA9 expression

In the training cohort, patients in the CA9-positive group showed a lower 5-year OS rate (43.1 vs 63.2 %, $p<0.001$; Fig. 4a) and lower 5-year RFS rate (39.9 vs 50.3 %, $p=0.004$; Fig. 4b) than patients in the CA9-negative group. In univariate analysis, both OS and RFS were significantly associated with CA9 positivity (for mortality: hazard ratio [HR]=1.70, 95 % confidence interval [CI]=1.38–2.11, $p<0.001$; for recurrence: HR=1.38, 95 % CI=1.10–1.71, $p=0.004$) (Tables 4 and 5). Moreover, in multivariate analysis, CA9 expression was an

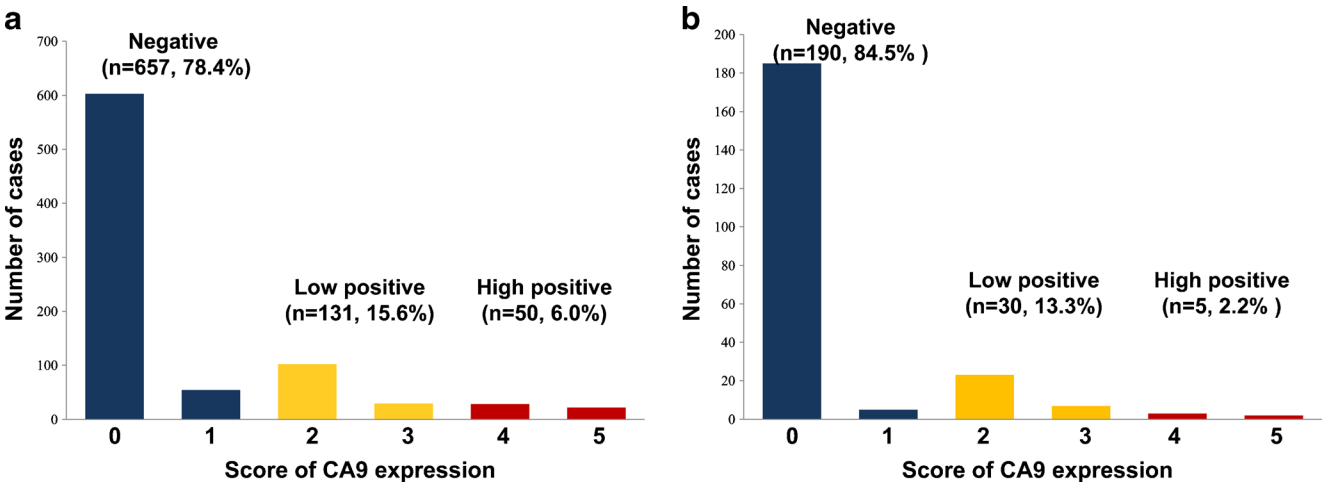

Fig. 3 Frequency distribution of the CA9 expression of 838 hepatocellular carcinoma patients in the training cohort (**a**) and 225 hepatocellular carcinoma patients in the validation cohort (**b**)

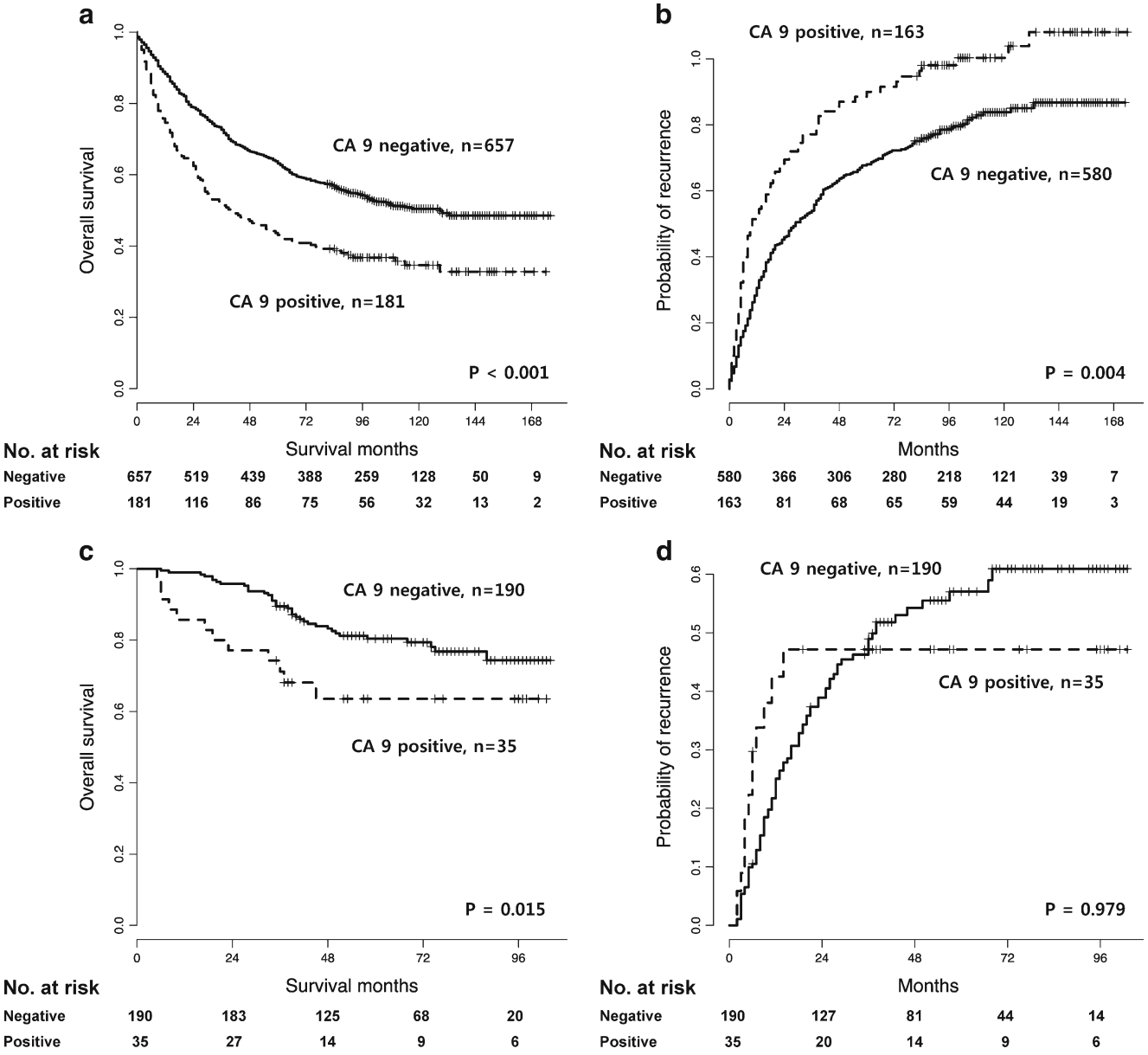

Fig. 4 Comparison of survival rates according to CA9 expression. Overall survival (**a**) and recurrence-free survival (**b**) are significantly poorer in CA9-postive patients in the training cohort. CA9 positivity is associated with a poorer overall survival (**c**), but not recurrence-free survival (**d**), in the validation cohort

independent prognostic marker for OS (HR=1.37, 95 % CI= 1.04–1.80, p=0.023), along with tumor size (>5 cm), microvascular invasion, PV or HV invasion, and Glisson capsule invasion (p<0.001, p<0.001, p<0.001, and p=0.003, respectively), but was not significantly associated with RFS (p=0.384) (Tables 4 and 5).

Validation cohort analysis

To confirm our results, CA9 expression was validated in an independent cohort of 225 early clinical stage patients who underwent hepatectomy at a later date. These patients had had no previous treatment (except for two patients who underwent PVE), had no PV or HV invasion (high AJCC T stage), and were not Child-Pugh class B or C. The mean age at the time of diagnosis was 55 years (range, 26–80 years), and 169 patients (75.1 %) were male (Table 2). During observation, 95 patients (42.2 %) experienced relapse, and the cause of all 50 patient deaths (22.2 %) was associated with HCC. The median OS and RFS was not reached. The estimated 5-year OS and RFS rates were 77.7 and 57.3 %, respectively.

The distribution of the immunoscore in the validation cohort was similar to that in the training cohort (Fig. 3). The characteristics of the validation cohort and correlations between CA9 expression and clinicopathological variables are summarized in Tables 2 and 3. There were 35 patients (15.6 %) in the CA9-positive group and CA9 positivity correlated with a tumor size larger than 5 cm (p=0.021) and a high Edmondson-Steiner grade (p<0.001). Patients in the

Table 3 Correlation between clinicopathological characteristics and CA9 expression

Characteristics	Training cohort			Validation cohort		
	CA9-negative (n=657)	CA9-positive (n=181)	p	CA9-negative (n=190)	CA9-positive (n=35)	p
Age			0.152			0.856
≤50 years	280 (42.6)	88 (48.6)		59 (31.1)	12 (34.3)	
>50 years	377 (57.4)	93 (51.4)		131 (68.9)	23 (65.7)	
Sex			0.829			0.737
Male	535 (81.4)	149 (82.3)		144 (75.8)	25 (71.4)	
Female	122 (18.6)	32 (17.7)		46 (24.2)	10 (28.6)	
Serum AFP[b]			<0.001[a]			0.094
≤100 ng/ml	368 (56.6)	68 (37.8)		113 (59.5)	15 (42.9)	
>100 ng/ml	282 (43.4)	112 (62.2)		77 (40.5)	20 (57.1)	
Hepatitis			0.131			0.311
NBNC	39 (5.9)	5 (2.8)		32 (16.8)	9 (25.7)	
HBV or HCV	618 (94.1)	176 (97.2)		158 (83.2)	26 (74.3)	
Liver cirrhosis			0.736			0.113
Absent	214 (32.6)	62 (34.3)		94 (49.5)	23 (65.7)	
Present	443 (67.4)	119 (65.7)		96 (50.5)	12 (34.3)	
Tumor diameter			<0.001[a]			0.021[a]
≤5 cm	441 (67.1)	91 (50.3)		146 (76.8)	20 (57.1)	
>5 cm	216 (32.9)	90 (49.7)		44 (23.2)	15 (42.9)	
Multiplicity			0.247			0.635
Solitary	560 (85.2)	161 (89.0)		176 (92.6)	31 (88.6)	
Multiple	97 (14.8)	20 (11.0)		14 (7.4)	4 (11.4)	
E-S grade			<0.001[a]			<0.001[a]
I or II	251 (38.2)	24 (13.3)		136 (74.6)	10 (28.6)	
III or IV	406 (61.8)	157 (86.7)		54 (25.4)	25 (71.4)	
Microvascular invasion			<0.001[a]			0.161
Absent	450 (68.5)	71 (39.2)		158 (83.2)	25 (71.4)	
Present	207 (31.5)	110 (60.8)		32 (16.8)	10 (28.6)	
PV or HV invasion[c]			<0.001[a]			-[d]
Absent	597 (91.8)	146 (82.0)		190 (100)	35 (100)	
Present	53 (8.2)	32 (18.0)		0 (0)	0 (0)	
Glisson capsule invasion			0.002[a]			>0.999
Absent	588 (89.5)	146 (80.7)		181 (95.3)	33 (94.3)	
Present	69 (10.5)	35 (19.3)		9 (4.7)	2 (5.7)	
Previous treatment			>0.999			>0.999
Absent	412 (62.7)	114 (63.0)		189 (99.5)	34 (97.1)	
Present	245 (37.3)	67 (37.0)		1 (0.5)	1 (2.9)	

AFP alpha-fetoprotein, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *E-S grade* Edmondson-Steiner grade, *PV* portal vein, *HV* hepatic vein

[a] Significance at the level of p<0.05

[b] Only 830 patients with available information on serum AFP in the training cohort

[c] Only 828 patients with available information on PV or HV invasion in the training cohort

[d] No statistics are computed because PV or HV invasion is a constant in the validation cohort

CA9-positive group had a lower 5-year OS rate (63.6 vs 80.4 %, p=0.015; Fig. 4c) than patients in the CA9-negative group. However, CA9 expression was not significantly associated with RFS (p=0.979) (Fig. 4d). In univariate and subsequent multivariate analysis, expression of CA9 was also an independent prognostic factor associated with reduced OS (HR=2.04, 95 % CI=1.05–3.96, p=0.035) (Table 4). However, in univariate analysis, CA9 was not significantly associated with RFS (p=0.984) (Table 5).

Pooled cohort analysis

For further analysis, a pooled cohort (n=1063) was created from the training and validation cohorts. Additional comparison of OS rates according to the degree of staining was performed in the pooled cohort. There were 161 patients (15.1 %) in the low CA9-positive group and 55 patients (5.2 %) in the high CA9-positive group. The CA9-positive group had a significantly worse OS than the low CA9-positive and negative groups (5-year OS: 39.3 vs 48.3 vs 66.9 %, respectively; p<0.001; Fig. 5). When the survival of the three groups was compared in pairs, a significant difference in survival was also observed between the CA9-negative and low CA9-positive groups (p<0.001), as well as between the low CA9-positive and high CA9-positive groups (p<0.001) (Fig. 5).

Discussion

This is the first large-scale study to evaluate the prognostic value of CA9 expression in HCC. In both a training cohort of 838 patients and a validation cohort of 225 patients, CA9 expression was proven to be a prognostic factor for OS after surgery for HCC. In addition, the high CA9-positive group had a poorer prognosis than the low CA9-positive group. Therefore, CA9 can be a useful and reliable marker for prognosis of HCC.

CA9 is a membrane-bound zinc metalloenzyme and by immunostaining predominantly expressed on the cell membrane [11, 23]. CA9 is overexpressed in various malignancies

Table 4 Univariate and multivariate analyses of overall survival according to the clinicopathological variables

Variables	Training cohort			Validation cohort		
	HR	95 % CI	p	HR	95 % CI	p
Univariate analysis						
Age > 50 years	1.16	0.96–1.41	0.116	0.83	0.47–1.49	0.550
Male	1.05	0.82–1.34	0.684	0.80	0.43–1.49	0.490
Serum AFP >100 ng/ml	1.30	1.07–1.57	<0.001[a]	1.19	0.68–2.08	0.534
Hepatitis B or/and C virus	1.03	0.68–1.55	0.882	0.99	0.49–1.99	0.992
Liver cirrhosis	0.96	0.79–1.17	0.723	0.70	0.39–1.24	0.227
Tumor diameter > 5 cm	2.11	1.75–2.56	<0.001[a]	1.96	1.11–3.46	0.020[a]
Multiple tumor	1.05	0.89–1.38	0.676	2.48	1.16–5.31	0.019[a]
E-S grade III or IV	1.68	1.35–2.08	<0.001[a]	1.21	0.68–2.14	0.511
Microvascular invasion	2.36	1.95–2.81	<0.001[a]	2.48	1.37–4.51	0.002[a]
PV or HV invasion	3.00	2.31–3.89	<0.001[a]	NA		
Glisson capsule invasion	2.28	1.77–2.92	<0.001[a]	3.68	1.55–8.71	0.003[a]
CA 9 expression	1.70	1.38–2.11	<0.001[a]	2.19	1.14–4.20	0.017[a]
Multivariate analysis						
Serum AFP > 100 ng/ml	0.89	0.72–1.10	0.295	NA		
Tumor diameter > 5 cm	1.65	1.34–2.04	<0.001[a]	1.39	0.74–2.60	0.297
Multiple tumor	NA			1.89	0.81–4.41	0.140
E-S grade III or IV	1.19	0.94–1.52	0.055	NA		
Microvascular invasion	1.69	1.37–2.10	<0.001[a]	1.86	0.95–3.63	0.067
PV or HV invasion	1.83	1.38–2.42	<0.001[a]	NA		
Glisson capsule invasion	1.48	1.13–1.93	0.003[a]	2.19	0.81–5.90	0.119
CA 9 expression	1.37	1.04–1.80	0.023[a]	2.04	1.05–3.96	0.035[a]

HR hazard ratio, *CI* confidence interval, *AFP* alpha-fetoprotein, *E-S grade* Edmondson-Steiner grade, *PV* portal vein, *HV* hepatic vein, *NA* not applicable

[a] Significance at the level of p<0.05

Table 5 Univariate and multivariate analyses of recurrence-free survival according to the clinicopathological variables

Variables	Training cohort[b]			Validation cohort		
	HR	95 % CI	*p*	HR	95 % CI	*p*
Univariate analysis						
Age >50 years	1.08	0.89–1.31	0.388	1.01	0.65–1.56	0.946
Male	1.16	0.89–1.50	0.252	1.19	0.73–1.94	0.463
Serum AFP >100 ng/ml	1.23	1.02–1.49	0.030[a]	0.97	0.64–1.47	0.913
Hepatitis B or/and C virus	0.90	0.59–1.31	0.626	1.22	0.71–2.08	0.468
Liver cirrhosis	0.92	0.75–1.12	0.423	0.96	0.64–1.44	0.870
Tumor diameter >5 cm	1.79	1.48–2.17	<0.001[a]	1.57	1.02–2.41	0.039[a]
Multiple tumor	0.80	0.59–1.08	0.147	2.04	1.09–3.83	0.025[a]
E-S grade III or IV	1.66	1.34–2.05	<0.001[a]	0.801	0.51–1.24	0.321
Microvascular invasion	1.57	1.30–1.91	<0.001[a]	1.69	1.04–2.72	0.031[a]
PV or HV invasion	1.47	1.08–1.98	0.012[a]	NA		
Glisson capsule invasion	1.92	1.47–2.49	<0.001[a]	4.40	2.19–8.83	<0.001[a]
CA 9 expression	1.38	1.10–1.71	0.004[a]	0.99	0.55–1.78	0.984
Multivariate analysis						
Serum AFP > 100 ng/ml	0.91	0.74–1.13	0.427	NA		
Tumor diameter >5 cm	1.59	1.28–1.96	<0.001[a]	1.39	0.85–2.25	0.179
Multiple tumor	NA			1.45	0.72–2.92	0.287
E-S grade III or IV	1.40	1.10–1.79	0.005[a]	NA		
Microvascular invasion	1.22	0.98–1.52	0.069	1.18	0.67–2.08	0.556
PV or HV invasion	0.94	0.68–1.30	0.725	NA		
Glisson capsule invasion	1.31	0.97–1.75	0.070	3.25	1.48–7.16	0.003[a]
CA 9 expression	1.10	0.87–1.40	0.384	NA		

HR hazard ratio, *CI* confidence interval, *AFP* alpha-fetoprotein, *E-S grade* Edmondson-Steiner, *PV* portal vein, *HV* hepatic vein, *NA* not applicable

[a] Significance at the level of $p<0.05$

[b] Only 743 patients with available information for relapse in the training cohort

invariably linked to the development of tumor hypoxia [12]. Hypoxia produces intracellular acidification and leads to a change in the behavior of tumor cells, allowing them to survive and adapt to hypoxic situations via the activation of genes related to angiogenesis, cell proliferation, and apoptosis inhibition, mediated by hypoxia-inducible factor 1 (HIF-1). *CA9* is one of the genes most strongly induced by hypoxia and plays an important role in pH regulation processes critical for tumor cell growth [10]. In response to hypoxia, HIF-1 directly activates transcription of the *CA9* gene and upregulates CA9 protein expression [21, 22, 24, 25].

For renal cell carcinoma, CA9 is a powerful and specific diagnostic marker [23]. CA9 expression is also known as a prognostic factor associated with an aggressive phenotype and worse outcome for many solid malignancies of various organs [15, 20, 25–28]. As in tumor tissue, an increased serum CA9 level indicates an unfavorable prognosis, such as higher stage in gastric cancer and frequent occurrence of postoperative relapse in clear cell renal cell carcinoma [29–32]. Kock et al. [32] found that the serum level of CA9 is correlated with intratumoral CA9 expression and that a high preoperative serum value is associated with poor prognosis in vulvar cancer. Therefore, they suggested that serum CA9 might be an easily assessable marker for stratifying patients for adjuvant therapy and, potentially, to monitor response [32].

In vitro studies have clearly demonstrated that CA9 stimulates the metastatic properties of cancer cells via CA9-induced acidification under hypoxic conditions, including decreased cell adhesion, increased cell motility, migration, and neovascularization, and protease activation [33]. CA9 expression is an indicator of metastatic propensity because CA9-expressing tumor cells exhibit highly metastatic behavior [33]. In addition, recent in vitro and in vivo studies revealed that carbonic anhydrase inhibitors derived from acetazolamides, ethoxzolamides, and benzenesulfonamides significantly inhibited tumor growth and metastasis formation [34–36]. These findings suggest a role for CA9 inhibitor as a novel drug for targeting metastatic carcinoma.

Kockar et al. [10] reported that hypoxia is a positive regulator of CA9 expression in HCC through four signal

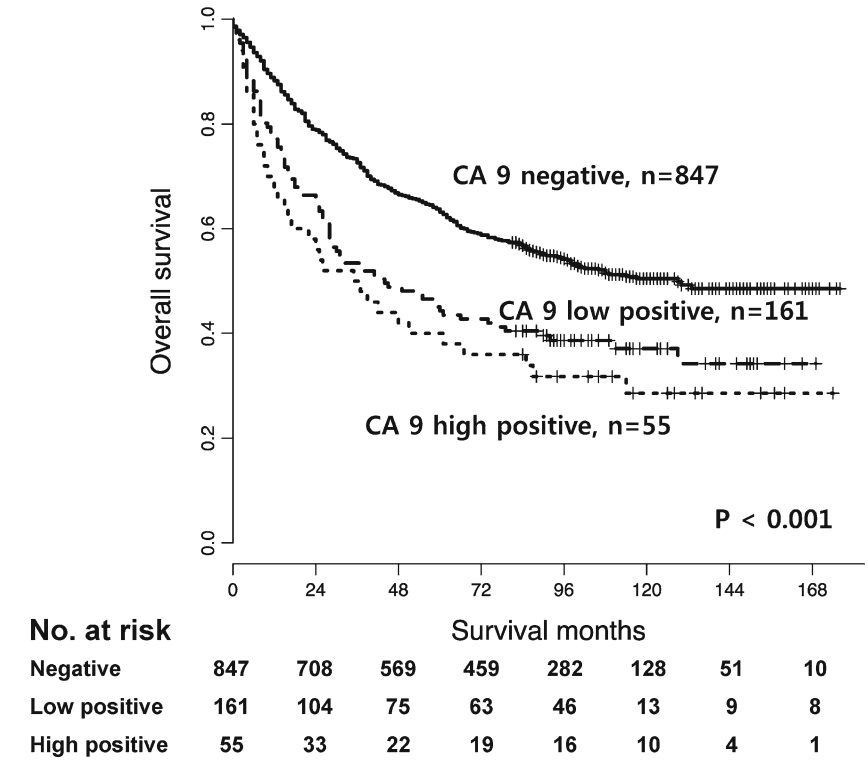

Fig. 5 Comparison of survival rates according to the degree of CA9 expression in the pooled cohort. There is a statistically significant difference in overall survival among the groups

transduction pathways, IL-1, IL-6, TNF-α, and TGF-β. Yu et al. [22] demonstrated that inhibition of hypoxia-induced CA9 enhances hexokinase II inhibitor-induced HCC apoptosis. However, the expression of CA9 in human tissues of HCC remains to be properly examined, and its expression has not been evaluated in terms of prognostic value. Although there was no correlation between CA9 immunoreactivity and clinicopathological variables in the previous study, tumoral CA9 intensity was related to E-cadherin downregulation, which has been frequently associated with invasiveness, metastasis, and poor prognosis in a variety of cancers [22]. These findings support the potential prognostic significance of CA9 expression in HCC and suggest that inhibition of CA9 might provide a new therapeutic approach for hypoxic HCC after local ablation [10, 22].

Because this was a retrospective study using a tissue microarray approach, additional studies are needed to conclusively determine if CA9 inhibitor might be a novel anticancer agent for the treatment of recurrent or inoperable HCC patients.

In conclusion, we studied the expression of CA9 and assessed potential clinical implications in a large number of HCC patients. These data indicate that CA9 expression is a poor prognostic factor in resectable HCC patients.

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

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