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Carbohydrate Antigen 19-9 Is a Prognostic Factor Which Correlates With HDAC1 and HIF-1α for Intrahepatic Cholangiocarcinoma

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Abstract. *Background/Aim: Carbohydrate antigen 19-9 (CA19-9) is a poor prognostic marker in intrahepatic cholangiocarcinoma (IHCC). Previous studies have shown a link between hypoxia and CA19-9 in cancer, and we have previously demonstrated a correlation between HDAC1 and HIF-1α in IHCC. Here, we evaluated the expression and correlation of CA19-9 with HIF-1 and HDAC in IHCC. Patients and Methods: This study included 62 patients with IHCC who underwent primary hepatectomy at our department. Clinicopathological characteristics were examined. Immunohistochemical expression of HIF-1 and HDAC1 in specimens was quantitatively evaluated. Results: Patients with high preoperative serum CA19-9 levels showed clinicopathological characteristics associated with tumour progression. High CA19-9 levels were associated with worse overall and recurrence-free survival. Univariate and multivariate analysis detected high CA19-9 levels as an independent poor prognostic factor for IHCC. Serum CA19-9 was significantly correlated with both HIF-1α and HDAC1 expression. Conclusion: High serum CA19-9 level is a poor prognostic factor for overall survival in IHCC and correlates with HIF-1α and HDAC1 expression.*

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Intrahepatic cholangiocarcinoma (IHCC) is a common secondary hepatic tumour (1). Currently, the only effective treatment for IHCC is curative resection. However, in Japan, patients with IHCC who are treated with curative resection still show poor prognosis (2).

Carbohydrate antigen 19-9 (CA19-9), also known as sialyl Lewis a (sLea) antigen or cancer antigen 19-9, is a cell surface marker (3) that is the most commonly validated tumour marker in several gastrointestinal cancers (2, 4-8). CA19-9 is a glycoprotein and cell adhesion molecule that binds E-selectin or P-selectin and its expression is related with metastasis and tumour embolism (9). CA19-9 also functions as a tumour marker for cholangiocarcinoma (10) and is a significantly poor prognostic marker in IHCC (4, 11, 12). Some studies have indicated that high serum CA19-9 level is a poor prognostic factor after curative resection (11) and chemotherapy (12). However, no reports have investigated the correlation between serum CA19-9 levels and other biomarkers in malignancies of digestive organs.

Hypoxia enables tumour cells to grow rapidly *via* hypoxia induced factor-1 (HIF-1) expression. The HIF-1 transcription factor regulates the expression of genes involved in critical pathways in angiogenesis, tumour growth and metastasis (13). Previous studies have shown that CA19-9 is upregulated in hypoxia conditions. Hypoxia induces upregulation of sLea (CA19-9) synthesis *via* transcription of some related glycogens. Expression of sLea in cancer cell clones also promotes hypoxia resistance and the development of haematogenous metastasis in advanced stage cancer (14). In addition, HIF-1 also supports the emergence of cancer stem cells (CSCs). We have previously reported that increased expression of HIF-1α is associated with increased tumour volume and poor prognosis in IHCC (15).

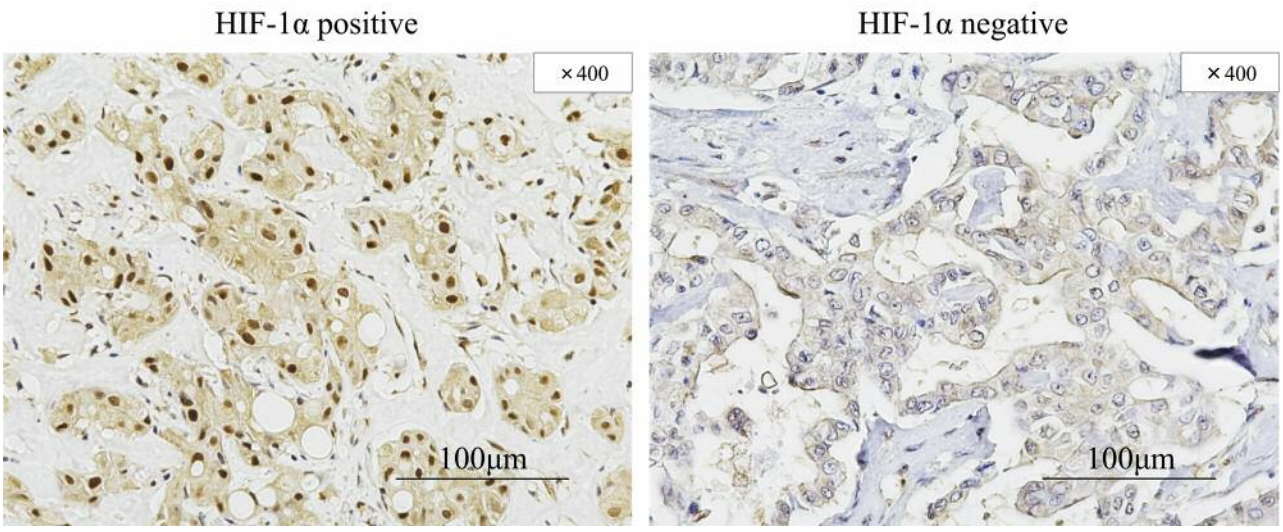

Figure 1. *Detection of HIF-1α in IHCC. HIF-1α expression is localized in cancer cell nuclei. Representative images of HIF-1α expression (positive and negative) in IHCC as determined by immunohistochemistry.*

Histone deacetylases (HDACs) play an important role in regulating gene expression through the control of histone acetylation status and chromatin remodelling. Recent studies have shown that HDAC1 and HIF-1α expression are significantly correlated with each other in pancreatic cancer (16). We have also previously reported the correlation between HDAC1 and HIF-1α in IHCC and cholangiocarcinoma cells (17) and demonstrated the correlation between HDAC1 and CSCs (18).

Based on these studies, we speculated that CA19-9 may correlate with HIF-1 and HDAC1 in IHCC. The aim of this study was to investigate the clinical significance of CA19-9 and its correlation with HIF-1α and HDAC1 expression in IHCC.

Patients and Methods

Patients. Sixty-two patients with IHCC were enrolled in this study. All patients underwent primary hepatectomy with curative intent from January 1994 to December 2016 in our department. After hepatectomy, patients with pathological stage III or IV received adjuvant chemotherapy. The clinical stage, curability, T-grade and N-grade were determined according to the Classification of Primary Liver Cancer by the Liver Cancer Study Group of Japan (19). T-grade was based on tumour diameter (>2 cm/≤2 cm), tumour number (≥2/<2) and intrahepatic vascular infiltration (presence/none). The pathological findings including tumour differentiation (well/others) and other findings were diagnosed by the Department of Pathology in Tokushima University hospital. All clinical information was available in the hospital information system of our hospital. This study was approved by the Institutional Review Board of the Tokushima University Graduate School (approved ID number 3541) and all patients provided written informed consent.

Immunohistochemistry. Fifty IHCC tumour specimens were available for immunohistochemistry. Four-micrometre-thick sliced specimens were deparaffinized and rehydrated and endogenous peroxidase was blocked. The slides were incubated in Protein Serum Free (Dako, Burlington, Canada) for 10 min to prevent nonspecific antigen binding. Samples were incubated with primary antibody for 1 h at room temperature. Primary antibodies included anti-human HIF-1α antibody (20960-1-AP, 1:100, Proteintech, Chicago, IL, USA) and anti-human HDAC1 antibody (sc-81598, 1:100; Santa Cruz, Paso Robles, CA, USA). The slides were incubated in secondary antibody EnVision Dual Link System-HRP (Dako) for 1 h at room temperature. Samples were developed in diaminobenzidine (Wako) and counterstained with Mayer's haematoxylin (No.3000-2, Muto Pure Chemicals Co. Ltd., Tokyo, Japan).

Stained slides were observed and evaluated by two pathologists. Both HIF-1α and HDAC1 expression were detected in the nucleus. HIF-1α positive expression was determined by counting the number of tumour cells in which 10% or more of the cells were positive (Figure 1), according to the previous report (17). HDAC1 expression was quantified by modified semiquantitative scoring as previously described (20) and scored as 0 (no staining), 1+ (weakly stained), 2+ (moderately stained) and 3+ (strongly stained). Samples with scores of 2+ or 3+ were defined as positive for HDAC1 expression (Figure 2).

Statistical analysis. Statistical calculations were performed using SPSS statistics version 24 64-bit Windows (IBM, Chicago, IL, USA). All clinical data were analysed by Mann-Whitney *U*-test or Fisher *U*-test. Overall survival (OS) and recurrence-free survival (RFS) were compared by Kaplan–Meier's curves and log-rank test. Univariate analysis was determined by log-rank test. Multivariate analysis was evaluated by Cox hazard model. *p*-Value less than 0.05 was considered to indicate statistical significance.

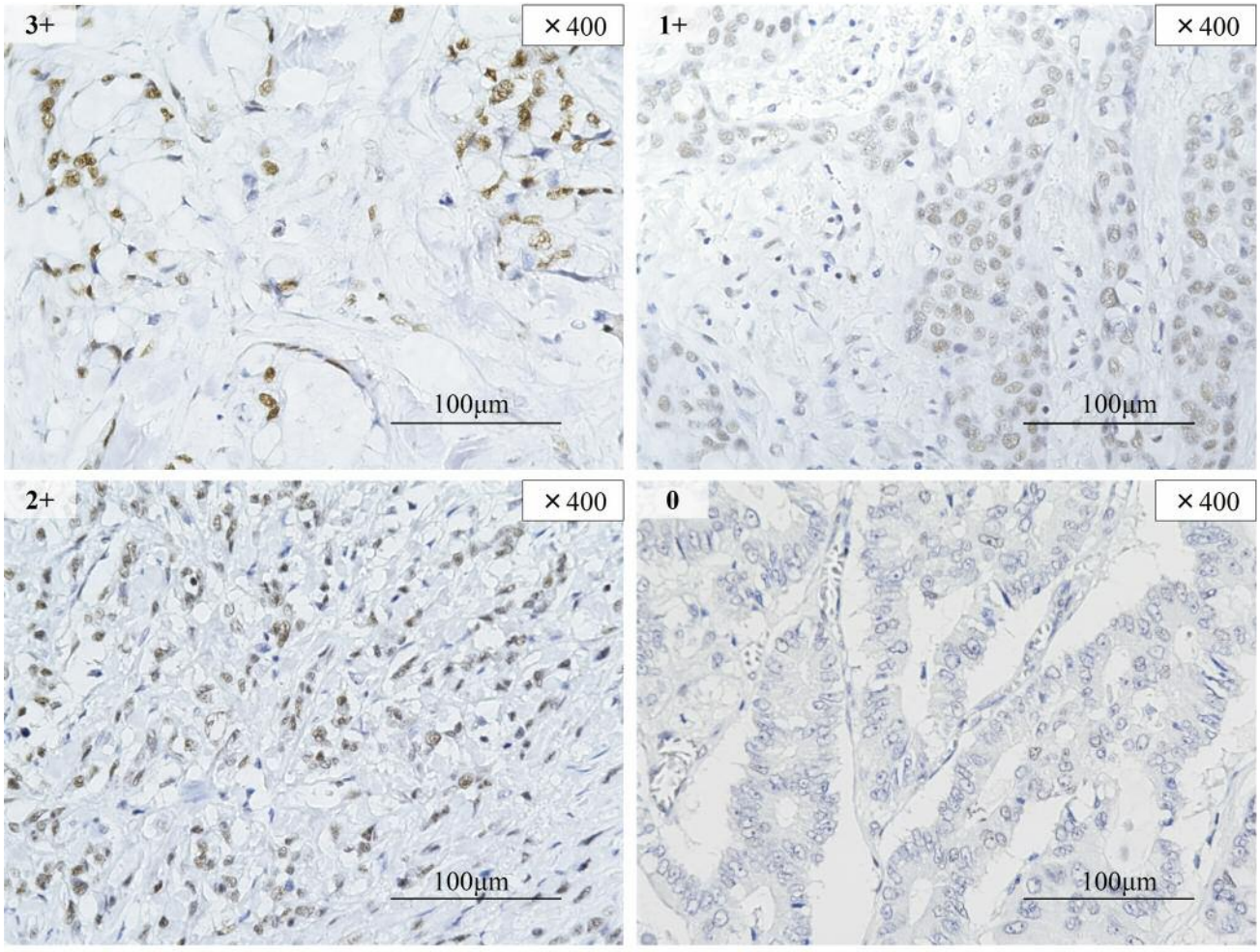

Figure 2. *Detection of HDAC1 in IHCC. HDAC1 expression is localized in cancer cell nuclei. Representative images of HDAC1 expression (0 to 3+) in IHCC as determined by immunohistochemistry.*

Results

CA19-9 is an independent poor prognostic factor in IHCC. This study included 62 patients with IHCC who underwent primary hepatectomy with curative intent at our department. The 62 patients were divided according to the preoperative serum CA19-9 level, as previously described (11), into the preoperative high serum CA19-9 group (>100 IU/ml, n=31) or the low serum CA19-9 group (<100 IU/ml, n=31). Table I shows the clinicopathological characteristics of both groups. The high CA19-9 group had significantly higher T grade (p=0.007) and more advanced clinical stage (p=0.043) than the low CA19-9 group. In pathological findings, the high CA19-9 group had more portal infiltration (p=0.033) compared with the low CA19-9 group. There were no significant differences in the other clinicopathological characteristics. These results suggested that high serum CA19-9 levels correlated with tumour progression in IHCC.

Kaplan–Meier curves of OS (Figure 3) and RFS (Figure 4) were evaluated in the two groups. The high CA19-9 group had significantly poorer prognosis than the low CA19-9 group in both OS (5-year OS 9.4% *vs*. 53.8%, p=0.002) and RFS (5-year RFS 10.8% *vs*. 27.0%, p=0.033). Univariate analysis detected T grade (p<0.001), lymph node metastasis (p<0.001), advanced clinical stage (p<0.001), pathological portal infiltration (p=0.001) and high CA19-9 levels (p=0.002) as significant poor prognostic factors for OS. Multivariate analysis detected only high CA19-9 level (p=0.033) as an independent poor prognostic factor for OS (Table II), and advanced clinical stage (p=0.016) was detected as an independent poor prognostic factor for RFS

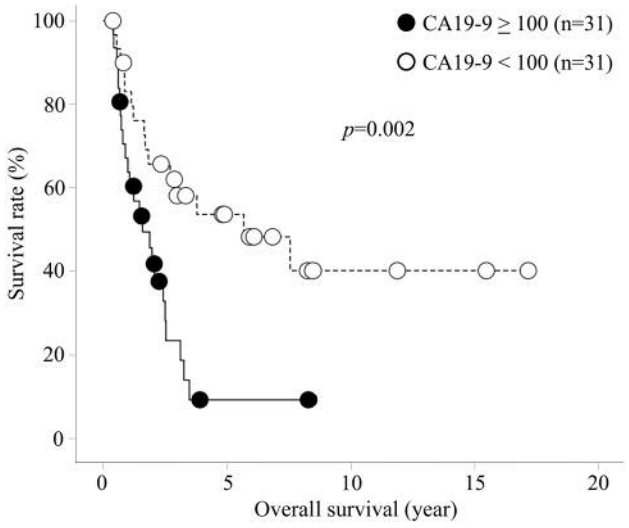

Figure 3. *Kaplan–Meier curves of overall survival according to serum CA19-9 levels. Closed circles, CA19-9 high (≥100 IU/ml) patients. Open circles, CA19-9 low (<100 IU/ml) patients. p-Value was calculated by the log-rank test.*

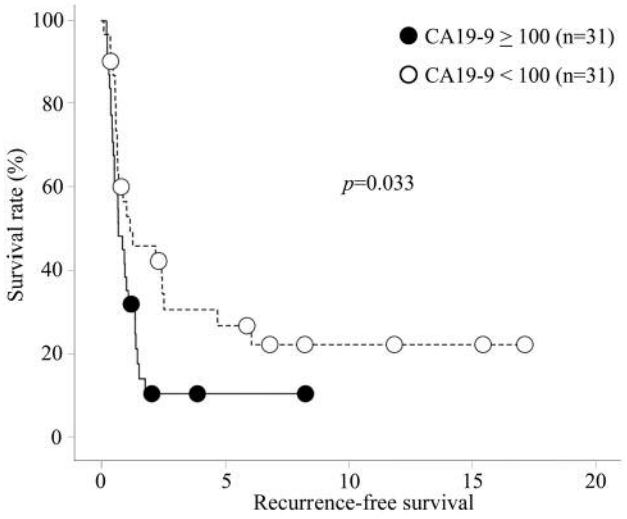

Figure 4. *Kaplan–Meier curves of overall survival according to serum CA19-9 levels. Closed circles, CA19-9 high (≥100 IU/ml) patients. Open circles, CA19-9 low (<100 IU/ml) patients. p-Value was calculated by the log-rank test.*

(Table III). These results demonstrate that preoperative serum CA19-9 level is an independent poor prognostic factor for OS in IHCC.

Both HIF-1α and HDAC1 expression correlated with serum CA19-9 level. Fifty of 62 specimens were available for evaluation in our dataset. HIF-1α-positive expression was detected in 23 IHCC patients. HDAC1 positive expression was detected in 19 IHCC patients. Figure 5 shows the correlations among HIF-1α, HDAC1 and serum CA19-9 levels. HIF-1α and CA19-9 were significantly correlated with each other (positive 62.5% *vs*. negative 33.3%, p=0.041) (Figure 5a). HDAC1 and CA19-9 were also significantly correlated with each other (positive 56.5% *vs*. negative 26.1%, p=0.036) (Figure 5b).

Representative IHCC case. The representative IHCC case was a 71-year-old female. She underwent posterior segmentectomy for treatment of a single IHCC in the S6 segment. Preoperative CA19-9 levels were high (879 IU/l) and CEA levels were within normal limits (4.5 ng/ml). The diameter of the tumour was 1.8 cm (Figure 6). In pathological findings, there was only microscopic vessel infiltration and no other pathological findings. Almost 6 months later, remnant liver recurrence occurred and the patient underwent right lobectomy and caudal lobe partial resection. However, soft tissue recurrence in the circumference of the inferior vena cava occurred 2 months after secondary surgery. The patient received chemotherapy (gemcitabine and cisplatin therapy) but died 18 months after primary surgery. Both HDAC1 and HIF-1α expression were positive.

Table I. *Clinicopathological characteristics of CA19-9 high and low patient groups.*

Factors	CA19-9 high (n=31)	CA19-9 low (n=31)	*p*-Value
Gender (M/F)	18/13	22/9	0.213
Age (y.o.)	68.4±1.8	68.7±1.7	0.836
Hepatic virus infection ((–)/HBV/HCV/combined)	24/4/3/0	19/8/3/1	0.405
CEA (ng/dl)	5.4 (0.8-340)	1.9 (0.6-2719)	0.069
Curability (A, B/C)	23/8	29/2	0.064
T (1, 2/3, 4)	15/16	5/26	0.007
N (0/1-3)	19/12	24/7	0.135
cStage (I, II/III, IV)	5/26	12/19	0.043
Tumour type (MF/MF±PI)	8/23	14/17	0.092
Differentiation (well/others)	7/24	9/22	0.386
Portal infiltration (–/+)	15/16	23/8	0.033
Venous infiltration (–/+)	26/5	27/4	1.000
Intrahepatic metastasis (–/+)	26/5	25/6	1.000

CA19-9 high: ≥100 IU/ml; CA19-9 low: <100 IU/ml; y.o.: years old; HBV: hepatitis B virus; HCV: hepatitis C virus; CEA: cancer embryonic antigen; MF: mass forming type; cStage: clinical stage; PI: periductal infiltrative type.

Table II. *Univariate and multivariate analysis of prognostic factors for overall survival.*

Factors	n/n	Univariate		Multivariate	
		5-year OS (%)	*p*-Value	HR	*p*-Value
Gender (M/F)	40/22	32.8/34.5	0.896		
Age (<60/>60)	10/52	45.7/29.9	0.488		
CEA (<5/>5))	39/22	37.7/27.3	0.340		
Curability (A, B/C)	52/10	37.0/11.4	0.073	1.100	0.827
T (1, 2/3, 4)	20/42	68.5/16.4	<0.001	1.008	0.853
N (0/1-3)	43/19	42.9/11.3	<0.001	1.663	0.178
cStage (I, II/III, IV)	17/45	75.8/17.7	<0.001	4.441	0.156
Tumour type (MF/MF + PI)	23/41	50.6/21.9	0.037	1.002	0.995
Differentiation (well/others)	16/46	32.8/33.4	0.703		
Portal infiltration (–/+)	38/24	42.4/17.7	0.001	1.505	0.265
Venous infiltration (–/+)	53/9	34.7/22.2	0.182		
Intrahepatic metastasis (–/+)	51/11	37.1/18.2	0.045	1.237	0.619
CA19-9 (<100/>100)	31/31	53.8/9.4	0.002	2.506	0.033

HR: Hazard ratio.

Table III. *Univariate and multivariate analysis of prognostic factors for recurrence-free survival.*

Factors	n/n	Univariate		Multivariate	
		5-year RFS (%)	*p*-Value	HR	*p*-Value
Gender (M/F)	40/22	14.6/27.2	0.479		
Age (<60/>60)	10/52	20.0/17.4	0.931		
CEA (<5/>5))	39/22	18.6/19.8	0.730		
Curability (A, B/C)	52/10	22.3/0	0.040	1.260	0.560
T (1, 2/3, 4)	20/42	50.1/2.8	<0.001	0.957	0.209
N (0/1-3)	43/19	26.4/0	<0.001	1.881	0.089
cStage (I, II/III, IV)	17/45	59.3/2.6	<0.001	8.746	0.012
Tumour type (MF/MF + PI)	23/41	37.1/7.1	0.027	1.160	0.678
Differentiation (well/others)	16/46	8.3/21.9	0.805		
Portal infiltration (–/+)	38/24	27.2/4.2	<0.001	1.783	0.093
Venous infiltration (–/+)	53/9	19.2/11.1	0.482		
Intrahepatic metastasis (–/+)	51/11	22.2/0	0.016	1.436	0.353
CA19-9 (<100/>100)	31/31	27.0/10.8	0.033	1.436	0.313

Discussion

This study identified serum CA19-9 level as an independent poor prognostic factor for IHCC. Serum CA19-9 levels correlated with both HIF-1α and HDAC1 expression. HIF-1α and HDAC1 tended to correlate with each other. These results showed that serum CA19-9 levels reflect the expression of HIF-1α and HDAC1.

HIF-1 is a transcriptional factor that is induced during hypoxia. HIF-1-regulated genes encode proteins that function in angiogenesis, metabolic reprogramming, extracellular matrix remodelling, epithelial-mesenchymal transition, motility, invasion, metastasis, cancer stem cell maintenance, immune evasion and resistance to chemotherapies (13, 21). Previous studies have indicated that HIF-1 may activate CSCs (22-24). We have also reported the correlation between HIF-1 expression and increased CSCs (24). Both HIF-1 and CA19-9 are upregulated during hypoxia (14).

Histone acetylation is a mechanism of epigenetic regulation of gene expression that is downregulated by HDAC family proteins. HDAC1 is a member of the class I HDAC molecules that have been examined as potential cancer therapeutic targets (25). In general, gene activation correlates with increased acetylated histones. Histone acetylation de-condenses the three-

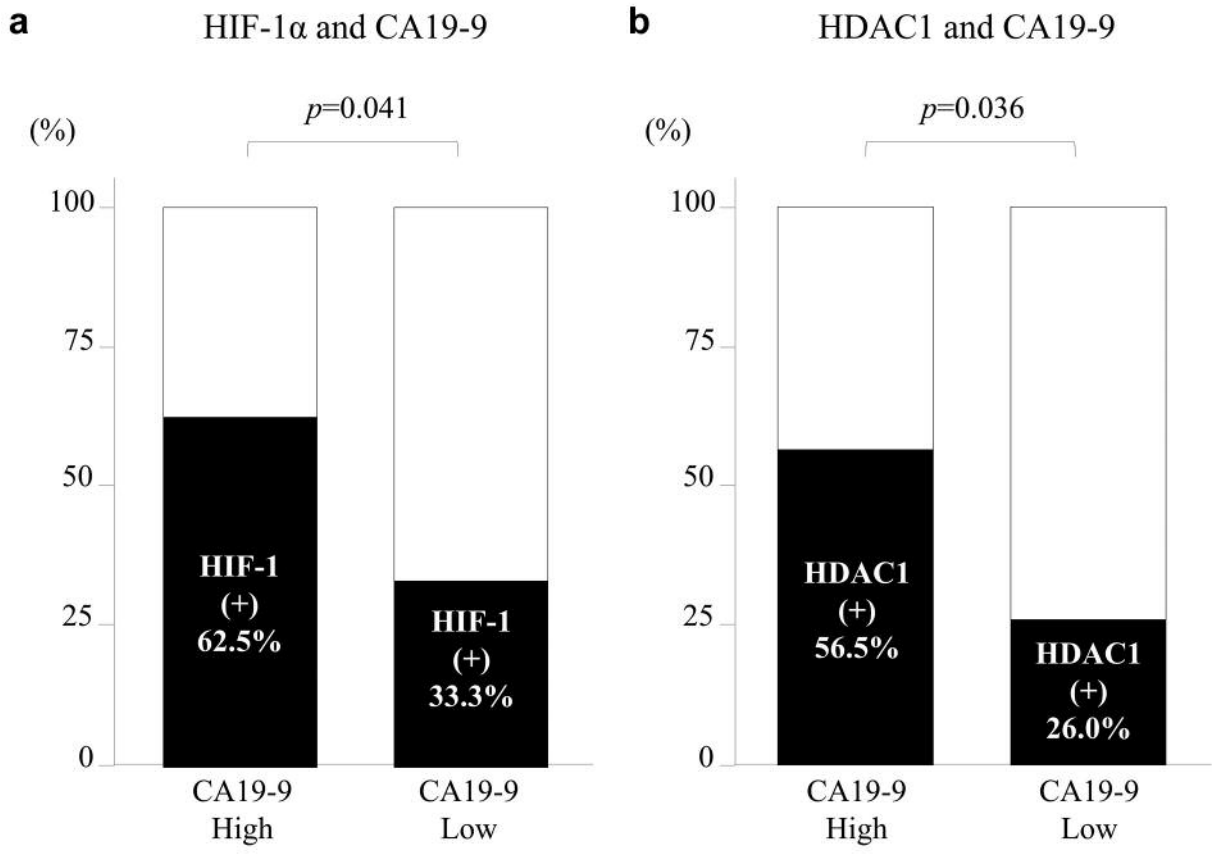

Figure 5. *Correlation between serum CA19-9 levels, HIF-1α and HDAC1 expression in IHCC. Both HIF-1α and HDAC1 expression significantly correlated with serum CA19-9 level.*

dimensional chromatin structure to enable transcriptional factors to bind to DNA. Histone deacetylation induces the condensation of chromatin, leading to inactive chromatin structure (25, 26). HDAC1 is necessary for the maintenance of CSCs. HDAC inhibitors including valproic acid have been significantly effective for CSCs phenotypes in ovarian and breast cancer (27, 28). We have also reported that HDAC1 expression is related to the existence of CSCs (18).

Our studies have reported the significance of the expression of HIF-1 and HDAC1 in gastroenterological cancer (15, 29-32). In colon cancer cells, hypoxic culture and HIFs induced the expression of sialic acid transporters fucosyltransferase and sialyltransferase, which are necessary for the expression of sLea and × antigen (33, 34). We have also previously demonstrated the relationship between HDAC-1 and HIF-1 expression in CSCs (18, 35). Taken together, our results suggest that hypoxia may induce HDAC1 and CA19-9 expression *via* HIF-1 upregulation in IHCC. Thus, serum CA19-9 levels might reflect the existence of CSCs. As shown in representative cases, some IHCC cases with high CA19-9 levels have worse prognosis because of early recurrence.

This study had several limitations. All 62 case specimens could not be evaluated in this study because samples were not available for all cases. Furthermore, we were unable to use the same anti-HDAC1 and anti-HIF-1α antibodies as in our previous report (17) as they have been discontinued. In addition, the immunohistochemistry methods in the current study were different from our previous report (17). However, our results showing that HDAC1 expression tended to correlate with HIF-1α expression were similar to our previous results (17). Also, downstream molecules of HDAC1 and HIF-1α, and CSC markers, including CD133 and NANOG, were not investigated. However, based on our previous results (15, 17, 18, 29, 30-32, 35), high levels of CA19-9 would associate with activation of CSCs.

In conclusion, we showed that high serum CA19-9 level was an independent poor prognostic factor and correlated with both HIF-1α and HDAC1 expression in IHCC. High serum CA19-9 levels might reflect the increase in CSCs *via* increased expression of HIF-1α and HDAC1 due to hypoxia, which are therapeutic molecular targets.

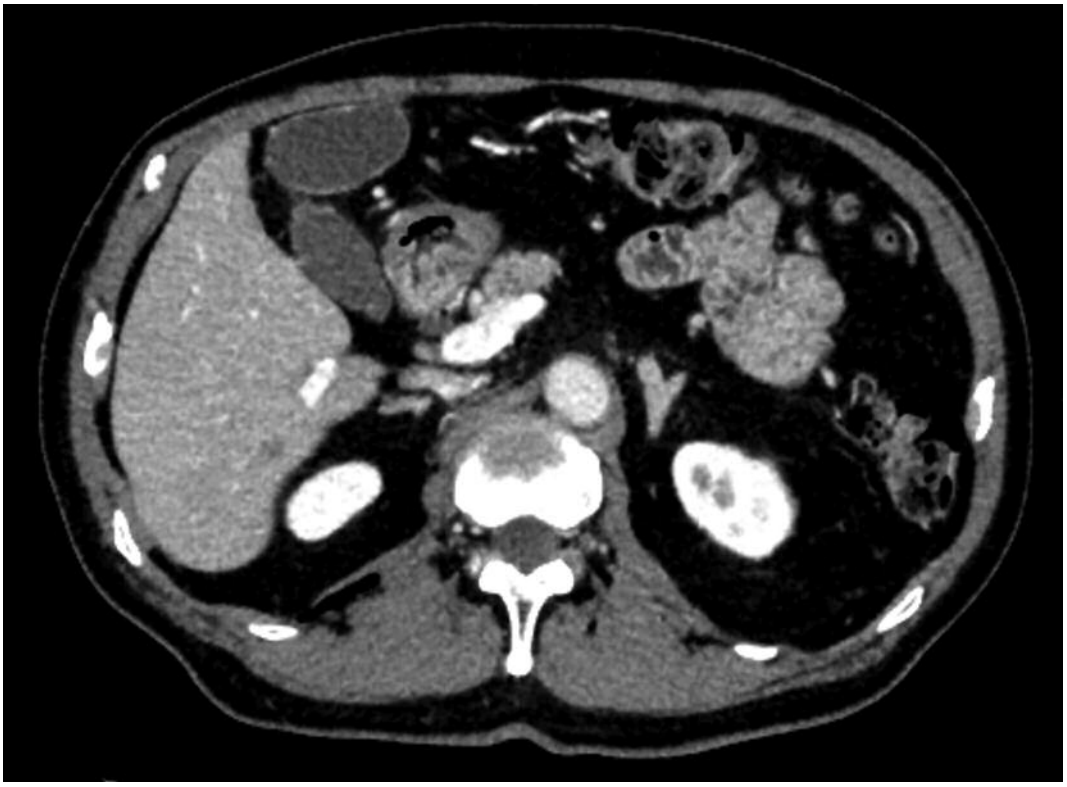

Figure 6. *Representative case with high CA19-9levels. Computed tomography imaging revealed a single IHCC of 1.8 cm in the S6 segment.*

Conflicts of Interest

The Authors declare no conflicts of interests regarding this study.

Authors' Contributions

S.O. conducted and performed the experiments, analysed data, and wrote the manuscript; M.S., Y.M. designed and planned the experiments; S.I. and T.I. advised in terms of the experimental techniques; Y.A. submitted the application for approval of the clinical research; Y.S., S.Y. and Y.W. checked the clinical and prognostic data in our dataset; S.Y. and Y.B. checked the specimen and performed the pathological evaluation; M.S. checked the experiments and edited the manuscript. All Authors reviewed the manuscript.

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