Impact of JMJD6 on intrahepatic cholangiocarcinoma

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Abstract. The association of Jumonji domain-containing 6 (JMJD6) with the prognosis of various types of cancer has been demonstrated, except in intrahepatic cholangiocarcinoma (ICC). The present study aimed to clarify the impact of JMJD6 on ICC. The liver specimens of 51 patients who underwent surgery for ICC were analyzed for JMJD6 expression using immunohistochemistry staining. The relationship between clinicopathological factors and JMJD6 expression was investigated. The cellular activity was also evaluated in JMJD6 knocked down cells with Transwell migration assay and viability assay. In the immunohistochemistry staining of clinical samples, high expression of JMJD6 was seen in 32 of 51 samples. High expression was also associated with improved overall survival (OS) and recurrence-free survival (RFS) (P=0.0033 and 0.048, respectively). Further analyses revealed that higher JMJD6 expression was one of the improved independent prognostic factors of OS and RFS. Expression of JMJD6 was knocked down in commercial culture cell lines of ICC, and RNA and protein were extracted to analyze the downstream gene expression using RNA-sequencing and western blotting. JMJD6 knockdown was associated with higher programmed death-ligand 1 (PD-L1) expression in RNA-sequencing and western blotting. In addition, PD-L1 expression was higher in JMJD6 low expression clinical samples when measured using immunohistochemistry staining. In conclusion, high expression of JMJD6 was an independent favorable prognostic factor of ICC. JMJD6 may influence the prognosis of ICC through the regulation of PD-L1 expression.

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Abbreviations: CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; ICC, intrahepatic cholangiocarcinoma; IHC, immunohistochemistry staining; JMJD6, Jumonji domain-containing 6; OS, overall survival; PD-L1, programmed death-ligand 1; RFS, recurrence-free survival; RT-qPCR, reverse transcription-quantitative PCR

Key words: JMJD6, PD-L1, cholangiocarcinoma, liver tumor

Introduction

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary hepatic malignancy, accounting for >5% of primary liver cancers, and the number of cases is increasing worldwide (1). The only potentially curative treatment of ICC is surgical resection, although some patients subsequently develop recurrence (2). Several combinations of systemic chemotherapy have shown to improve patients' survival; however, the etiology and pathogenesis of ICC remain poorly understood (3-5).

Jumonji domain-containing 6 (JMJD6), a member of the Jumonji C domain-containing family of proteins, was originally identified as a phosphatidylserine receptor (PSR) on cell surface (6). Subsequent studies have demonstrated that JMJD6 is located in the nucleus and has demethylase and hydroxylase activities toward histone and non-histone proteins (7,8). There is growing evidence to indicate that JMJD6 overexpression is associated with advanced clinicopathological stage, increased aggressiveness, and poor survival in various types of cancer; however, the impact of JMJD6 on ICC has not been reported yet (9).

Tumor immunology is a hot topic describing the interaction between the immune system and tumor cells. Understanding these interactions is important for the development of novel therapies for cancer. Immune checkpoint inhibitors function by reducing the suppression of T cells, especially $CD8^+$ T cells, to improve tumor-specific responses (10,11). An increasing number of reports describe the relationship between $CD8^+$ T cells and ICC prognosis. Our institution has shown that decreased microvessel density is related to worse prognosis, and Asahi *et al* also reported that high CD8 count could be an improved prognostic factor of ICC (12,13).

In this study, we assessed the clinical relevance and prognostic significance of JMJD6 expression in ICC. We also aimed to reveal the possible mechanism and the relationship between JMJD6 and the tumor immunological environment via *in vitro* JMJD6 knockdown studies.

Materials and methods

Patients and tumor samples. We retrospectively examined patients with primary ICC who underwent surgical resection at the Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University. Fifty-three patients with ICC who were diagnosed between May 1998 and August 2017 were eligible for inclusion in the study. The patients provided the written consent for the use of their tissues for future scientific research at the time of collection. Paraffin-embedded specimens were retrieved from the Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University. The analyzed clinicopathological features included the age at surgery, sex, pathological stage (8th edition AJCC/UICC staging manual), microvascular invasion, laboratory data, and the clinical course of each patient (14). This study was approved by the Clinical Research Ethics Committee of Kyushu University Hospital according to the Ethical Guidelines of the Japanese Government (approval no. 30-578).

Immunohistochemistry (IHC). Formalin-fixed, paraffin-embedded tissue sections from patients with ICC were immunostained for JMJD6 (PSR H-7, sc-28348, Santa Cruz Biotechnology, Inc.; 1:100) and PD-L1 (clone SP142, Spring Bioscience; 1:100). Positive JMJD6 nuclear expression was defined as nuclear staining of ≥40% (Fig. 1). The programmed death-ligand 1 (PD-L1) expression on the membrane and cytoplasm was defined as positive when the percentage of positive cells was ≥5% of ICC cells (15). Immunohistochemical evaluations were independently performed by two observers (Y.K. and K.Y.) who were blinded to the clinical backgrounds of the patients. If the difference between evaluations was >10%, the evaluations were repeated. The findings of the two observers were averaged and considered final. The capture of microscopic images and quantitative analyses were undertaken on the NanoZoomer platform (Hamamatsu Photonics), and we ensured that the results matched with the observers' results.

Cell lines. The cholangiocarcinoma cell lines, SSP-25 and HuH-28, were obtained from Riken Bioresource Center, Tsukuba, Japan. The cell lines were originally isolated from intrahepatic cholangiocarcinoma specimens obtained from surgical resection of Japanese adult patients. The SSP-25 cell line was authenticated by STR profiling (supplemental document). The cells were incubated at 37˚C and 5% CO_2 in RPMI media (Thermo Fisher Scientific, Inc.) supplemented with heat-inactivated 10% fetal bovine serum and penicillin-streptomycin.

JMJD6 siRNA transfection. The siRNA transfection was performed as previously described (16). Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect the cells with siRNA (17). The siRNAs were obtained from Dharmacon, Inc. (siJMJD6-1 CAT# J-010363-10-0002, sequence=GGAGAGCACUCGAGAUGAU, siJMJD6-2 CAT# J-010363-12-0002, sequence=GGUAUAGGAUUUUGAAGCA, Control (non-targeting pool) CAT# D-001810-10-05). To facilitate transfection, the cells per well were incubated in 10% FBS containing RPMI; subsequently, they were plated to 40% confluence on a 6-well plate during transfection. We mixed 150 μl of Opti-MEM and 9 μl of RNAiMAX and subjected the mixture to incubation for 5 min at room temperature. In another tube, 10 pM siRNA in 3 ml of Opti-MEM and 150 μl of Opti-MEM were combined. Subsequently, we added siRNA solution to the diluted RNAiMAX reagent, and 250 μl of the prepared siRNA/RNAiMAX mixtures per well was used for incubation at room temperature for 25 min. Afterward, the $2.5x10^5$ cells per well and the solution were combined. They were incubated for 6 h at 37˚C, and the mixture was replaced with RPMI with 10% FBS. The cells were incubated for 72 h in maximum, and the transfection efficiency was monitored every 24 h using western blotting and real-time polymerase chain reaction (PCR) in order to optimize the appropriate incubation time, and was decided to be 48 h.

Transwell migration assay and viability assay. Transwell migration assay: A total of $4x10^4$ SSP-25 cells resuspended in 250 μl RPMI were placed on an 8.0-μm Transparent PET Membrane (Corning Inc.). The chamber was placed in a 24-well plate containing 750 μl RPMI and 10% FBS. After incubation at 37˚C overnight, migrating cells were stained with Diff-Quik (KACLaS) and were counted manually in five random microscopic fields at x200 magnification and quantified using ImageJ software (https://imagej.net/). The viability of the cells was examined by the CellTiter-Glo (CTG) assay (Promega) according to the manufacturer's instructions. Briefly, the cells were plated in 96-well plates with enough number of cells to 100% confluent in 24 h. The luminescence was read and quantified in 24 h with Multiskan GO spectrophotometric microplate reader (Thermo Fisher Scientific, Inc.).

RNA extraction and sequencing. RNA was extracted using the Maxwell(R) RSC simplyRNA tissue kit (Promega). Whole transcriptome sequencing was applied to RNA samples using the Illumina HiSeq 3000 platform in a 100-bp single-end mode. Sequenced reads were mapped to the human reference genome sequence (hg19) using TopHat version 2.0.13 in combination with Bowtie 2 version 2.2.3 and SAMTools version 1.0. The number of fragments per kilobase of exon per million mapped fragments was calculated using Cuffnorm version 2.2.1. RNA-Seq data were calculated as the fold change between samples with two-tailed Student's t-test ($P<0.1$) using the Subio Platform and Subio Basic Plug-in (v1.20; Subio, Inc.). Thresholds were set at a fold change of 2.0 and P-values of <0.05. Raw data of this study were submitted to Gene Expression Omnibus (accession no. GSE171974).

Reverse transcription-quantitative PCR (RT-qPCR). One microgram of total RNA was converted to cDNA using the SuperScript III First-Strand Synthesis Supermix (Thermo Fisher Scientific, Inc.) with oligo-dT primers as per manufacturer's instructions. Quantification was determined using

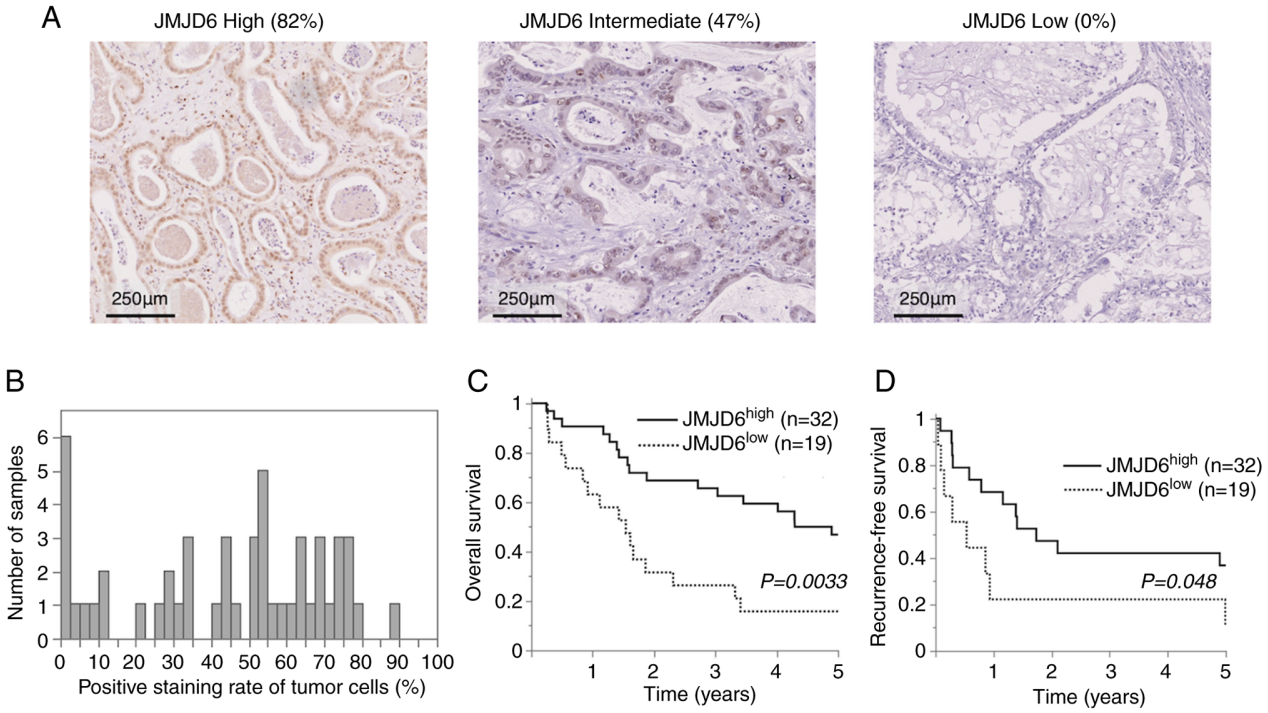

Figure 1. JMJD6 expression in clinical samples. (A) Representative images of high, intermediate and low JMJD6 expression in stained tissues (scale bar, 250 μm). (B) Number of samples for each staining rate and >35% of samples are marked as high expression. (C) Survival analysis of JMJD6 with overall survival. (D) Survival analysis of JMJD6 with recurrence-free survival curve. JMJD6, Jumonji-domain containing 6, MVI, microvascular invasion.

the ΔΔCt method relative to a β-actin control. The qRT-PCR primers used were as follows: JMJD6 Hs00397095_m1 and β-actin Hs01060665_g1. Assays were performed using the TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, Inc.) (18,19).

Western blot assay. Western blotting was performed as previously described (19). Briefly, whole-cell lysis was performed in RIPA buffer containing protease inhibitors (Nacalai Tesque). Proteins were separated by polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. After blocking with blocking buffer supplied in the iBind Western System (Thermo Fisher Scientific, Inc.), membranes were incubated with the primary antibody. Monoclonal antibodies for PD-L1 (1:1,000, E1L3N; Cell Signaling Technology), JMJD6 (1:200; Santa Cruz Biotechnology, Inc.), and β-actin (1:1,000; Cell Signaling Technology) were used. The JMJD6 antibody was derived from mouse, and the other antibodies were derived from rabbit. Horseradish peroxidase-conjugated secondary antibodies for mouse (1:1,000; Abcam) and rabbit (1:2,000; Abcam) were used. Primary and secondary antibodies were simultaneously applied on the rockers of the iBind Western System after setting membranes on a paper filter. Antibody binding was detected by enhanced chemiluminescence assays, and each band was detected using an Amersham Imager 600 (GE Healthcare Life Sciences).

Statistical analysis. Statistical analysis was performed with JMP Pro 16.1.0 statistical software. Comparisons of categorical and continuous variables were performed using the Chi-square test and Student's t-test or the Mann-Whitney U test, respectively. A P-value of <0.05 was considered significant. Cumulative overall survival (OS) and recurrence-free survival (RFS) rates were calculated using the Kaplan-Meier method, and differences between curves were evaluated using the log-rank test.

Results

JMJD6 expression in ICC specimens. Patients with ICC comprised 34 males and 17 females at an age range of 33-82 years (median=60). JMJD6 expression was analyzed in ICC specimens. Immunohistochemical analysis revealed that JMJD6 expression was predominantly noted in the nuclei (Fig. 2A and B). The percentage of stained tumor cells was analyzed, and the mean value was selected as the cut-off point to obtain comparable subgroup sizes.

Association of JMJD6 expression with clinicopathological features. The relationship between JMJD6 expression and clinicopathological factors in patients with ICC was evaluated (Table I). High JMJD6 expression was observed in 32 of 51 patients (62.7%). JMJD6 expression was not significantly associated with clinicopathological factors, except for older age in low-expression samples (65.6 vs. 58.7 years, P= 0.043).

JMJD6 expression and survival analysis. According to univariate analysis, low JMJD6 expression was significantly associated with poor overall survival (OS, P=0.0033) (Fig. 1C) and recurrence-free survival (RFS, P=0.048) (Fig. 1D).

Univariate analysis revealed that JMJD6 expression, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) were unfavorable predictors for

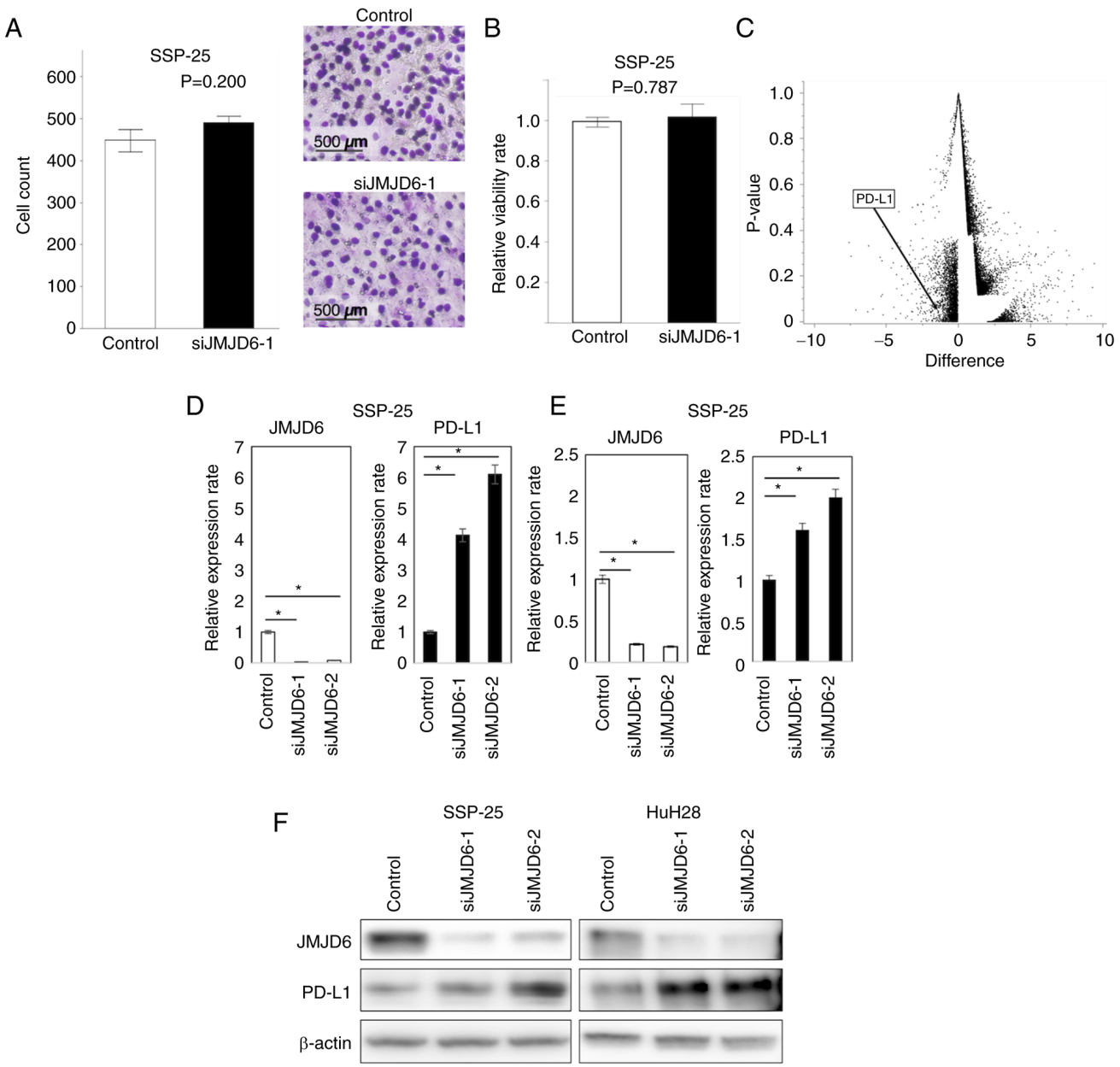

Figure 2. Expression of JMJD6 and PD-L1 in ICC cell lysates. (A) Migration assay with SSP-25 cells. Left: The bar chart of number of migrated cells. Right: The representative images of migration (scale bar, 500 μm). (B) Cell viability assay with SSP-25. (C) Volcano plot of RNA-sequencing results of SSP-25. (D) Bar chart of PD-L1 and JMJD6 expression. In the lysates of JMJD6-knockdown cells, PD-L1 was significantly overexpressed. (E) RT-qPCR of JMJD6-knockdown SSP-25 cell lysate. RT-qPCR demonstrated that PD-L1 expression was upregulated in the JMJD6-knockdown samples. (F) Western blotting of ICC cells after JMJD6-knockdown. The ICC cells showed the same expression patterns as the RNA analysis. *P<0.05. RT-qPCR, reverse transcription-quantitative PCR; ICC, intrahepatic cholangiocarcinoma; JMJD6, Jumonji-domain containing 6; si, short interfering; PD-L1, programmed death-ligand 1.

OS. In the multivariate analysis, low JMJD6 expression and high CA19-9 were revealed to be the independent worse prognostic factors for OS (Table II). In the analyses regarding RFS, univariate analysis showed that low JMJD6 expression, low serum albumin level, high carcinoembryogenic antigen (CEA), and microvascular invasion were unfavorable predictors for RFS. In the multivariate analysis, low JMJD6 expression, low serum albumin level and high carcinoembryogenic antigen (CEA) were independent worse prognostic factors of RFS (Table III). The analyses were also performed in the subgroups of peripheral and perihilar ICC and did not reveal any specific differences (data not shown).

In vitro JMJD6 expression, cellular assays and RNA sequencing. To explore the importance of JMJD6 in ICC, JMJD6 was knocked down in SSP-25 cells. The migration assay (Fig. 2A) and the viability assay (Fig. 2B) did not show any significant difference between JMJD6-knockeddown cells and the control. RNA sequencing revealed 91 genes whose expression were positively altered and 167 negatively altered genes in JMJD6-depleted cells (Fig. 2C and D, Table SI). Among those, we focused on immunology-related genes to evaluate the impact of tumor immunology on the prognosis of ICC. Notably, JMJD6 knockdown increased the expression of PD-L1. An increase in PD-L1 expression under JMJD6 depletion was also identified using RT-PCR (Fig. 2E). Western

Table I. Clinicopathological factors and JMJD6 expression in IHC.

Factors (Category)	JMJD6 High expression (n=32)	JMJD6 Low expression (n=19)	P-value
Median age, years (IQR)	58.7 (55-63)	65.6 (60-71)	0.043[a]
Sex			0.68
Male (%)	22 (69)	12 (63)	
Female (%)	10 (31)	7 (37)	
Albumin (g/dl)	4.11 (3.97-4.27)	4.03 (3.84-4.23)	0.49
Total bilirubin (mg/dl)	0.72 (0.31-1.13)	1.31 (0.78-1.84)	0.081
Tumor size (mm)	44.9 (37.3-52.4)	46.6 (37.3-55.9)	0.77
Solitary/Multiple	23/9	10/9	0.16
Microvascular invasion	16/14	13/5	0.20
Perihilar/Peripheral	12/20	10/9	0.29
Differentiation (well/mod/por)	10/20/2	9/8/1	0.42
CEA (ng/ml)	3.37 (1.88-4.86)	3.12 (1.18-5.06)	0.84
CA19-9 (U/ml)	1349 (0-6,946)	7,778 (863-14,693)	0.15

[a]P<0.05. CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; JMJD6, Jumonji-domain containing 6.

Table II. Univariate and multivariate Cox proportional hazard analyses of overall survival in patients with ICC. Bold numbers indicate statistically significant correlations (P<0.05).

	Univariate analysis		Multivariate analysis	
Factors	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
JMJD6, low expression	2.73 (1.36-5.48)	0.0047[a]	3.47 (1.62-7.45)	0.0014[a]
Age ≥60 (year)	1.20 (0.60-2.37)	0.6070		
Sex, male	1.39 (0.66-2.91)	0.3890		
Albumin ≤3.5 (g/dl)	1.87 (0.45-7.85)	0.3917		
Total bilirubin ≥2.0 (mg/dl)	2.99 (0.67-13.4)	0.1522		
CEA ≥5 (U/ml)	2.76 (1.18-6.46)	0.0189[a]	1.52 (0.61-3.81)	0.3671
CA19-9 ≥50 (U/ml)	2.08 (1.01-4.28)	0.0461[a]	2.72 (1.25-5.89)	0.0114[a]
Tumor size ≥5-cm (cm)	1.98 (0.99-3.97)	0.0530		
MVI, positive	1.94 (0.91-4.12)	0.0845		
Location, perihilar	1.26 (0.63-2.51)	0.5108		
Number of tumors >1	1.95 (0.97-3.91)	0.0593		

[a]P<0.05. CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; ICC, intrahepatic cholangiocarcinoma; JMJD6, Jumonji-domain containing 6; MVI, microvascular invasion.

blotting also revealed similar increase of PD-L1 expression in ICC cells after JMJD6 knockdown (Fig. 2F).

PD-L1 in clinical samples. To investigate the PD-L1 expression in clinical specimens, we performed IHC for PD-L1 in the same samples used for the IHC for JMJD6 (Fig. 3A). The result showed that 34 of 51 samples were positive for PD-L1. The rate of high PD-L1 expression was higher in the low JMJD6 group and vice versa (Fig. 3B; P=0.025). We also performed the survival analysis of PD-L1 expression, but it did not show any significant results (P-value for OS=0.3070, P-value for PFS=0.0687, data not shown).

Discussion

Patients with ICC have a poor prognosis, and curative treatments, such as surgical resection, are limited to early-stage disease. Systemic therapy options and their effectiveness are also limited. In contrast to the dramatic decrease of hepatocellular carcinoma owing to the development of virological treatment and newly emerged systemic therapy options, ICC treatment is limited and still requires further investigation (20).

In this study, we showed that the epigenetic regulator JMJD6 is a favorable prognostic factor for ICC and that JMJD6

Table III. Univariate and multivariate Cox proportional hazard analyses of recurrence-free survival in patients with ICC.

	Univariate analysis		Multivariate analysis	
Factors	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
JMJD6, low expression	2.20 (1.13-4.26)	0.0206[a]	2.33 (1.16-4.68)	0.0179[a]
Age ≥60 (year)	1.30 (0.67-2.50)	0.4388		
Sex, male	1.73 (0.83-3.60)	0.1428		
Albumin ≤3.5 (g/dl)	3.45 (1.03-11.5)	0.0448[a]	5.18 (1.45-18.5)	0.0113[a]
Total bilirubin ≥2.0 (mg/dl)	3.69 (0.84-16.2)	0.0835		
CEA ≥5 (U/ml)	4.10 (1.70-9.88)	0.0016[a]	3.80 (1.53-9.46)	0.0041[a]
CA19-9 ≥50 (U/ml)	1.58 (0.79-3.14)	0.1973		
Tumor size ≥5 (cm)	1.98 (0.99-3.97)	0.0530		
MVI, positive	2.32 (1.12-4.78)	0.0231[a]	1.97 (0.92-4.21)	0.0804
Location, perihilar	0.94 (0.55-2.06)	0.8586		
Number of tumors >1	1.93 (0.97-3.81)	0.0592		

[a]P<0.05. CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; ICC, intrahepatic cholangiocarcinoma; JMJD6, Jumonji-domain containing 6; MVI, microvascular invasion.

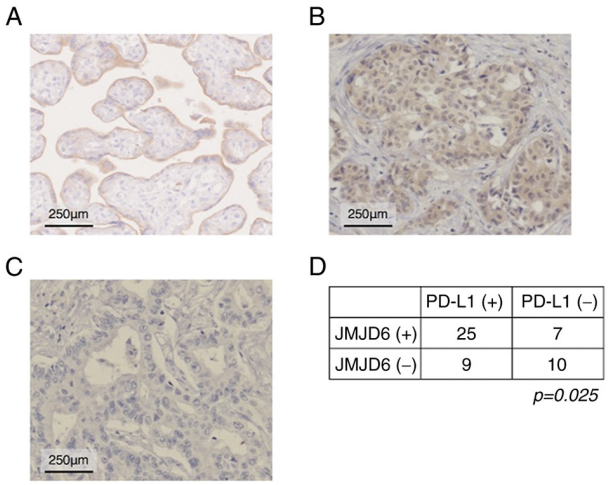

Figure 3. PD-L1 expression in ICC clinical samples. (A) Immunohistochemistry staining of PD-L1 with placenta as a positive control. (B) Representative images of positive staining. (C) Negative staining. (D) Relationship between positive JMJD6 and PD-L1 staining (scale bar, 250 μm). ICC, intrahepatic cholangiocarcinoma; JMJD6, Jumonji domain-containing 6; PD-L1, programmed death-ligand 1.

is a possible regulator of PD-L1 expression. A mechanism that can explain these results is that JMJD6 modifies the promoter region of PD-L1 and inhibits PD-L1 expression. JMJD6 demethylates histones and other proteins, and it promotes or inhibits gene expression depending on which amino acid and on what site they are located. Also, PD-L1 expression is reported to be upregulated through epigenetic regulation with histone demethylases (21). Thus, JMJD also has the possibility to regulate the expression of PD-L1.

The relationship between JMJD6 and tumor has previously been reported as an unfavorable prognostic factor in various cancers, including breast cancer, colon cancer, oral squamous cancer, melanoma, and hepatocellular carcinoma. However, to the best of our knowledge, the role of JMJD6 in ICC has not been investigated (22-24).

Our results showed that JMJD6 is a good prognostic factor, in contrast to past reports. As JMJD6 is an epigenetic modifier, the influence of the protein varies depending on what type of protein to regulate, e.g., CDK4 in HCC, p53 in colon cancer. Therefore, we performed RNA sequencing to elucidate the genes regulated by JMJD6, which resulted in the identification of 171 candidate genes. Our institution has previously reported the impact of tumor immunology on cancer prognosis (12,25,26); thus, we focused on immune-related genes. We found that PD-L1 mRNA levels increased in response to JMJD6 knockdown, indicating that JMJD6 regulates PD-L1 expression. This inverse relationship between JMJD6 and PD-L1 expression was confirmed by PCR, western blotting, and IHC.

Although *in vitro* experiments have revealed the connection between JMJD6 and PD-L1, this was not sufficient to explain that PD-L1 expression is controlled by JMJD6, and the key to connect them is the epigenetic modification. CD274, the gene encoding PD-L1, is located on chromosome 9p24.1. In this region, genomic regulation has been proven to upregulate PD-L1 expression, resulting in immune escape (27). Among these types of regulation, H3K4me3 is upregulated by MLL1, an H3K4 methylation-specific histone methyltransferase in pancreatic cancer (28). The present data suggest that a similar mechanism is possibly activated to mediate PD-L1 expression.

PD-L1 induces cancer cell immune evasion by binding to the PD-1 receptor on activated T cells, which results in tolerance of tumor-reactive T cells, rendering tumor cells resistant to CD8+ T cells (29). In the current study, although the positivity of PD-L1 was not sufficient to demonstrate any correlation with the prognosis of ICC, the high JMJD6 expression, which inversely reflected PD-L1 expression, impacted the prognosis of ICC. Also, the use of immune checkpoint inhibitor combined with the conventional

systemic therapy has emerged to be effective in prolonging the survival of biliary tract cancer patients. Thus, JMJD6 is a potential biomarker to prove the susceptibility of ICI in each individual.

This study has several limitations. First, the current study was derived from only one institution and the number of samples was small. Second, we did not perform any epigenetic experiments to directly prove the mechanism, by analysis with chromatin immunoprecipitation (ChIP) for example. Also, our cellular experiments only focused on viability and migration, and we did not perform the ones regarding apoptosis or cell cycle, which were reported in previous JMJD6 reports. Therefore, there is a room for future research about these factors. Additionally, the current research only presented the *in vitro* experiments and clinical sample study. Further studies using *in vivo* tumor mouse models and anti-PD-L1 agents are required to obtain more persuasive evidence.

In conclusion, JMJD6 is an independent favorable prognostic factor for ICC and is a candidate target protein for the treatment of ICC, focusing on the tumor microenvironment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YKF and SI carried out the molecular studies, participated in the sequence alignment and drafted the manuscript. KY carried out the evaluation of IHC. TF and DO performed the RNA sequencing. SI, TT and NH participated in the design of the study. YO, TY and MM conceived of the study and participated in its design and coordination and helped to draft the manuscript. YKF and SI confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Clinical Research Ethics Committee of Kyushu University Hospital according to the Ethical Guidelines of the Japanese Government (approval no. 30-578). The patients provided the written consent for the use of their tissues for future scientific research at the time of collection.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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