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Decreased expression of HDAC8 indicates poor prognosis in patients with intrahepatic cholangiocarcinoma

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ABSTRACT

Background: Intrahepatic cholangiocarcinoma (ICC) is a highly malignant primary tumor in the liver, and the rates of incidence and mortality are rapidly increasing globally. Histone deacetylase 8 (HDAC8) is a transcriptional regulator and is associated with tumorigenesis of several tumor types. This study aimed to evaluate the correlation between HDAC8 expression and clinicopathological parameters in ICC patients.
Methods: ICC tissues and corresponding nonmalignant bile duct tissues were obtained from 60 patients. HDAC8 and Ki-67 expression were evaluated by immunohistochemistry staining. HDAC8 expression and the clinicopathological features and prognosis of the patients were analyzed. The mRNA level of HDAC8 in ICC was further analyzed using data from The Cancer Genome Atlas (TCGA).
Results: The expression of HDAC8 were lower in ICC tissues (39/60, 65%) than in the corresponding nonmalignant bile duct tissues (54/60, 90%) ($P=0.001$). Low HDAC8 expression in ICC was significantly associated with lymph node metastases (47.6% vs. 17.9%, $P=0.015$). In addition, the positive cells rate of HDAC8 was statistically and negatively correlated with the Ki-67 index in ICC lesions ($r = -0.7660$, $P < 0.001$). Importantly, the overall survival rate and recurrence-free survival rate in ICC patients with low HDAC8 expression were lower than those with high HDAC8 expression ($P=0.008$ and $P=0.011$, respectively).
Conclusions: Decreased HDAC8 expression in ICC is related to poor prognosis, and HDAC8 may be an independent prognostic indicator of ICC patients after curative resection.

Introduction

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver cancer. In the past few decades, the incidence and mortality of ICC have rapidly increased worldwide [1–5]. ICC patients has no symptom at the early stage which makes it difficult to recognize [6,7]. Although metabolic disorders, including diabetes or obesity, chronic liver disease, such as chronic hepatitis B and C, cholestasis, primary sclerosing cholangitis, and parasitic liver flukes, and lifestyle or environmental factors, such as consumption of alcohol and smoking, are reported to be risk factors of ICC, the pathogenesis of ICC remains largely unknown [8–12]. Hepatectomy is still the best and most effective treatment for ICC [13]; however, recurrence after surgery is common [14,15].

Distinct from other members of the class I histone deacetylases (HDACs), HDAC8 is a unique member that lacks the C-terminal protein-binding domain. HDAC8 can remove acetyl moieties from lysine near the N termini of various histones and nonhistone proteins. The malleability of the surface facilitates HDAC8 undergoing conformational changes to accommodate different substrates [16]. HDAC8 activity is also negatively regulated by the phosphorylation of Ser39, which is close to the active site of HDAC8 [16]. HDAC8 plays diverse roles in biological functions. HDAC8 represses the expression of homeobox transcription factors in mice, playing an important role in skull morphogenesis [17]. Deficiency of HDAC8 results in inefficient dissolution of the cohesin complex and sister chromatid separation, therefore impairing mitosis [18]. HDAC8 maintains long-term hematopoietic stem cell and leukemia stem cell activity via regulating tumor suppressor p53 activity [19,20]. In addition, HDAC8 is associated with some special nonhistone

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proteins. HDAC8 can enhance the transcription of estrogen-related receptor α and bind cortical actin-binding protein to control muscle contraction [21,22].

HDAC8 dysregulation also plays critical roles in tumorigenesis [23]. HDAC8 is often overexpressed in highly malignant cancers, such as childhood cancer neuroblastoma, and high HDAC8 expression statistically correlates with poor overall survival and recurrence-free survival [24]. Inhibition of HDAC8 induces apoptosis in T-cell-derived lymphomas and leukemic cells [25]. Moreover, deregulation of HDAC8 is frequently observed in urothelial cancer [26]. It has been reported that overexpression of HDAC8 promotes tumor growth and inhibits apoptosis in hepatocellular carcinoma (HCC) [27], however, the origin of the ICC and HCC is from bile duct cells and hepatocytes respectively, and the tumorigenesis mechanism is different. The correlation between HDAC8 expression and the clinical significance of ICC remains unclear. In this study, we assessed the expression patterns and clinicopathological prognostic significance of HDAC8 in ICC patients.

Methods

Tissue samples and histopathology

Our study consisted of 60 patients who underwent curative-intent partial hepatectomy from July 2009 to September 2015 for ICC at West China Hospital, Sichuan University. All patients did not undergo radiotherapy or chemotherapy before hepatectomy. All pathological information was independently reviewed by two experienced pathologists. ICCs were histologically graded as well, moderately, and poorly differentiated adenocarcinoma according to their morphology as described before [28]. Tumor staging was classified according to the TNM staging of malignancies as defined by the American Joint Committee on Cancer (8th edition).

The pathological characteristics, including sex, age at diagnosis, the number of tumor nodules, HBV infection, tumor size, lymph node metastases, histologic grade, serum CA19-9 levels (ng/mL), vein invasion and TNM stage, were recorded. The study was reviewed and approved by the Ethics Committee of West China Hospital, Sichuan University, and was conducted in accordance with the protocol approved by the *Declaration of Helsinki*. Informed consent was obtained from every patient in the study.

Western blotting

The ICC samples were lysed in RIPA lysis buffer, and protein was extracted by centrifugation (12,000 rpm) at 4 °C for 15 min. A BCA protein assay kit (Merck, Darmstadt, Germany) was used to measure the total protein concentration. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. After blocking in 5% nonfat milk for 60 min, the membranes were incubated with rabbit anti-human HDAC8 (sc-365,620, Santa Cruz Biotechnology, USA) at 0.2 μg/mL or mouse anti-human GAPDH (AB2302, Millipore, Billerica, USA) at 0.2 μg/mL at 4 °C overnight. ECL reagent was used for chemiluminescence detection.

Immunohistochemistry staining

Paraffin-embedded (4 μm) sections of liver specimens (fixed in 4% paraformaldehyde) were stained with hematoxylin/eosin (HE) or various antibodies. In brief, the paraffin specimens were sectioned continuously, which were then dewaxed with xylene and hydrated in graded alcoholic solutions. EDTA at pH 9.0 was used to recover antigen with two rounds of 8 min of microwaving. The tissue slides were incubated with 3% H_2O_2 for 15 min to inactivate endogenous peroxidase and with goat serum for 30 min to avoid nonspecific protein binding. After the tissue slides were incubated with rabbit anti-human HDAC8 at 0.7 μg/mL or rabbit anti-human Ki-67 (7B11, Thermo) at 1 μg/mL at 4 °C overnight, an anti-rabbit IgG detection kit (Dako, Tucson, Arizona, USA) was used to demonstrate the immunochemistry staining according to the instruction manual. We obtained digitalized immunohistochemical images using a Nano Zoomer Digital Pathology C9600-01 Virtual Slide Light microscope scanner (Hamamatsu Photonics, Hamamatsu, Shizuoka-ken, Japan) by Hamamatsu and using NDP View software, version 2.5 (Hamamatsu Photonics, Hamamatsu).

Scoring system for immunohistochemistry

We used a semiquantitative assessment to score the expression of HDAC8 as described previously [29]. The method consists of measuring the staining intensity and the percentage of positive cells. The staining intensity was evaluated using 4 grades (0, negative; 1, weak; 2, moderate; and 3, strong). The percentage of positive cells was evaluated using 5 grades (0, ≤5%; 1, 6%–25%; 2, 26%–50%; 3, 51%–75%; and 4, >75%). The total score was calculated by multiplying the grade of positive cells by the intensity grade. Total scores <4 were considered low expression of HDAC8, while total scores ≥4 were considered high expression of HDAC8. The Ki-67 index was evaluated as the ratio of the positive cancer cells, which yielded 4 grades (1, ≤2%; 2, 3%–19%; 3, 20%–36%; and 4, >36%). A Ki-67 index below 19% was defined as low expression, and an index over 19% was defined as high expression according to the previous report [30].

Statistical analysis

All statistical analyses were carried out using SPSS 18.0 (version 18.0; SPSS Inc., Chicago, IL, USA). The relationship between HDAC8 expression and the corresponding clinicopathological parameters in ICC patients were analyzed using a Chi-square test or Fisher's exact test. Pearson correlation was used to analyze the correlation between HDAC8 expression and Ki-67 index in ICC patients. Recurrence-free survival was defined from the date of surgery to the date of tumor recurrence or the follow-up deadline. Overall survival was determined from the date of surgery to the date of death from cancer or the follow-up deadline. Survival analysis was assessed by the Kaplan-Meier method, and survival curves were analyzed by the log-rank test. Cox regression was used to analyze the relationship between HDAC8 expression and clinicopathological prognosis in ICC patients. A $P < 0.05$ was regarded as statistically significant.

Results

Clinical and pathological characteristics of patients

Among the ICC patients included in this study, there were 34 males (56.7%) and 26 females (43.3%). The median age was 54 (range 29–81) years. Half of the patients had tumors over 6 cm at the maximum diameter. Twenty-four patients (40%) were positive for hepatitis B surface antigen (HBsAg+). The ICC clinicopathological characteristics are presented in Table 1. Among the included cases, 19 (31.7%) were moderately differentiated and all the rest (68.3%) were poorly differentiated, respectively.

HDAC8 is frequently decreased in ICC tissues

Tissues diagnosed with ICC through HE and immunohistochemistry staining were used to assess the expression of HDAC8. In hepatocytes and biliary tract epithelium cells of nonmalignant tissues,

Table 1
Clinicopathological characteristics of ICC patients and the relation with HDAC8 level and Ki-67 expression.

Characteristics	*n*	HDAC8 expression		*P* value	Ki-67 expression		*P* value
		Low	High		Low	High	
Tissue				**0.001**			**<0.001**
ICC	60	21	39		16	44	
Adjacent	60	6	54		55	5	
Age (yr)				0.515			0.370
≥60	28	11 (52.4%)	17 (43.6%)		9 (56.3%)	19 (43.2%)	
<60	32	10 (47.6%)	22 (56.4%)		7 (43.8%)	25 (56.8%)	
Sex				0.090			0.255
Male	34	15 (71.4%)	19 (48.7%)		11 (68.8%)	23 (52.3%)	
Female	26	6 (28.6%)	20 (51.3%)		5 (31.3%)	21 (47.7%)	
Tumor size (cm)				0.787			1.000
<6	30	11 (52.4%)	19 (48.7%)		8 (50.0%)	22 (50.0%)	
≥6	30	10 (47.6%)	20 (51.3%)		8 (50.0%)	22 (50.0%)	
Tumor nodules				0.767			0.705
Solitary	47	16 (76.2%)	31 (79.5%)		12 (75.0%)	35 (79.5%)	
Multiple	13	5 (23.8%)	8 (20.5%)		4 (25.0%)	9 (20.5%)	
HBsAg				0.740			0.153
Positive	24	9 (42.9%)	15 (38.5%)		4 (25.0%)	20 (45.5%)	
Negative	36	12 (57.1%)	24 (61.5%)		12 (75.0%)	24 (54.5%)	
Lymph node metastases				**0.015**			0.518
Positive	17	10 (47.6%)	7 (17.9%)		3 (18.8%)	14 (31.8%)	
Negative	43	11 (52.4%)	32 (82.1%)		13 (81.3%)	30 (68.2%)	
Vein invasion				0.185			**0.041**
Positive	12	2 (9.5%)	10 (25.6%)		6 (37.5%)	6 (13.6%)	
Negative	48	19 (90.5%)	29 (74.4%)		10 (62.5%)	38 (86.4%)	
TNM stage				0.172			0.967
I/II	41	12 (57.1%)	29 (74.4%)		11 (68.8%)	30 (68.2%)	
III/IV	19	9 (42.9%)	10 (25.6%)		5 (31.3%)	14 (31.8%)	
CA19-9 (ng/mL)				0.176			0.559
<40	30	13 (61.9%)	17 (43.6%)		7 (43.8%)	23 (52.3%)	
≥40	30	8 (38.1%)	22 (56.4%)		9 (56.3%)	21 (47.7%)	
Histologic grade				0.395			0.550
Well differentiation	0	0	0		0	0	
Moderate differentiation	19	5 (23.8%)	14 (35.9%)		6 (37.5%)	13 (29.5%)	
Poor differentiation	41	16 (76.2%)	25 (64.1%)		10 (62.5%)	31 (70.5%)	

ICC: intrahepatic cholangiocarcinoma.

Table 2
Univariate analysis of recurrence-free survive and overall survival in ICC.

Variables	Recurrence-free survival		*P* value	Overall survival		*P* value
	HR	95% CI		HR	95% CI	
HDAC8 expression	2.281	1.015–5.127	**0.046**	2.380	1.061–5.338	**0.035**
Ki-67 expression	0.564	0.265–1.199	0.136	0.611	0.291–1.284	0.194
Age	0.931	0.465–1.865	0.841	1.117	0.573–2.177	0.745
Sex	0.678	0.316–1.453	0.317	0.957	0.459–1.993	0.906
Tumor size	1.261	0.675–2.358	0.467	1.190	0.648–2.188	0.575
Tumor nodules	1.389	0.675–2.858	0.373	1.528	0.743–3.142	0.249
HBsAg	1.096	0.545–2.203	0.798	0.801	0.404–1.587	0.525
Lymph node metastases	3.495	1.406–8.688	**0.007**	4.315	1.722–10.813	**0.002**
Vein invasion	0.690	0.268–1.781	0.443	0.841	0.326–2.170	0.721
CA19-9	2.412	1.128–5.155	**0.023**	1.863	0.924–3.757	0.082
TNM stage	1.596	0.732–3.479	0.240	1.747	0.853–3.575	0.127
Histologic grade	1.022	0.501–2.082	0.953	0.823	0.415–1.635	0.579

ICC: intrahepatic cholangiocarcinoma; HR: hazard ratio; 95% CI: 95% confidence interval.

HDAC8 is expressed mainly in the nucleus, and is also slightly distributed in the cytoplasm. Semiquantitative assessment of HDAC8 showed that HDAC8 was extensively and highly expressed in the nonmalignant tissues, and 54 cases (90%) had a score ≥4. In the malignant tissue, on the contrary, HDAC8 expression was low in 21 cases (35%) with a score <4 (Fig. 1). Ki-67-positive cells were rarely observed in the nonmalignant duct epithelium. In contrast, frequent mitotic cells were detected in cancer tissues. Among the included cases, 16 (27%) with a positive rate of Ki-67 ≤19% were grouped into Ki-67-low cases, and the rest were regarded as Ki-67-high cases (Fig. 1). Western blotting analysis showed that the level of HDAC8 protein expression was lower in ICC tissues than in the corresponding nonmalignant tissues (Fig. 2A and B). TCGA data showed no alteration in HDAC8 mRNA levels in 89% (32/36) of ICC tissues (Fig. 2C).

Relationship between HDAC8 expression and clinicopathological parameters

By comparing the HDAC8 status with various clinicopathological parameters of ICC patients, we found that low HDAC8 expression was more frequent in lymph node metastases (47.6% vs. 17.9%, $P = 0.015$). Other clinicopathological characteristics, including sex, age, tumor size, HBsAg, vein invasion, histologic grade, TNM stage, or the levels of CA19-9, were not statistically associated with HDAC8 expression (Table 1). Intriguingly, the positive cells rate of

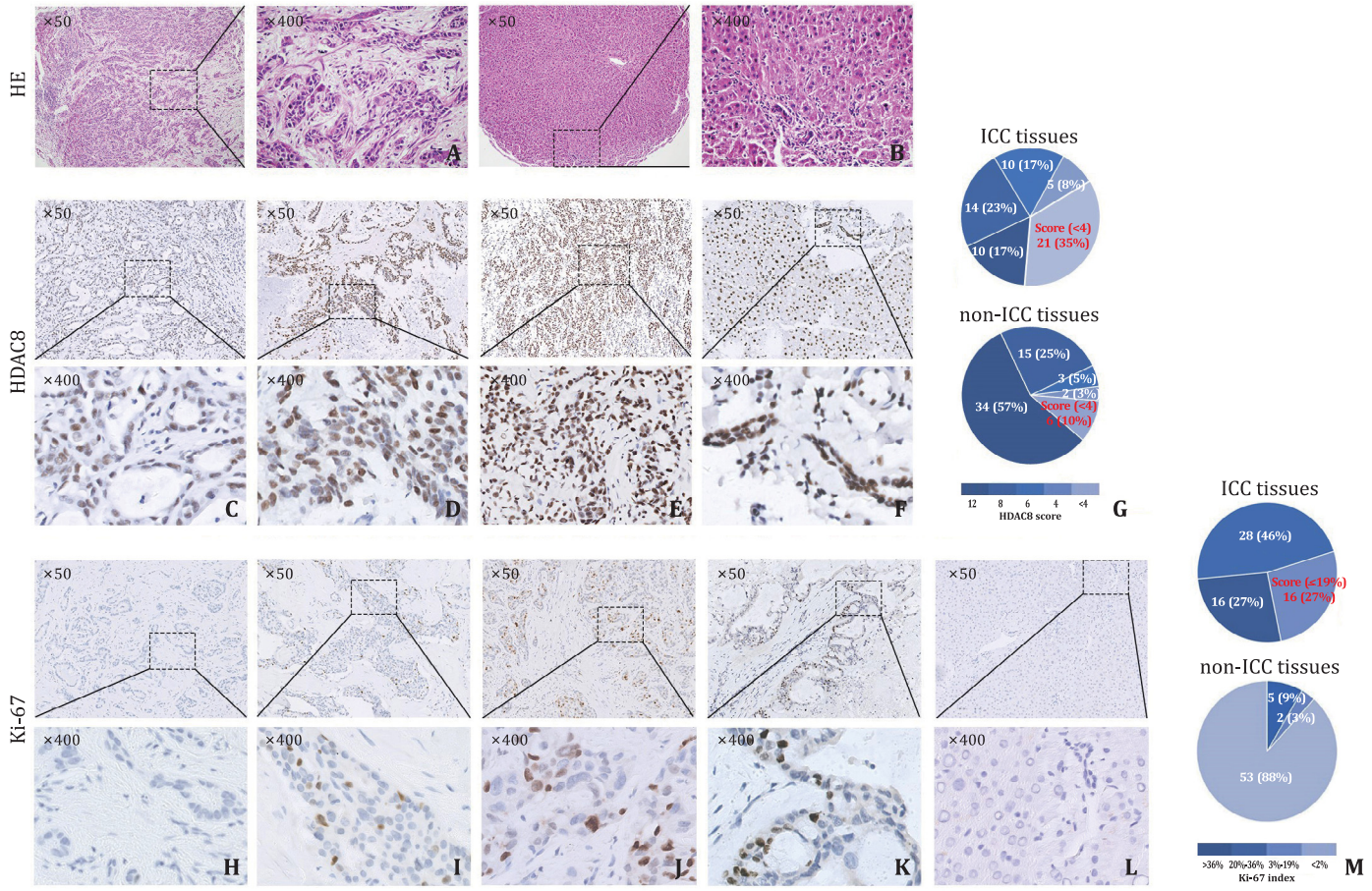

Fig. 1. Representative HE and immunohistochemistry staining of HDAC8 and Ki-67 of ICC and corresponding tissues. **A:** HE staining of ICC tissues; **B:** HE staining of corresponding nonmalignant bile duct tissues. **C:** weak staining intensity of HDAC8 (grade 1) in ICC; **D:** moderate staining intensity of HDAC8 (grade 2) in ICC; **E:** strong staining intensity of HDAC8 (grade 3) in ICC; **F:** strong staining intensity of HDAC8 (grade 3) in adjacent tissues. **G:** Semiquantitative assessment of HDAC8 showed that 21 cases (35%) had a score <4 in ICC, and 6 cases (10%) had a score <4 in nonmalignant tissues. **H:** no immunoreactivity of Ki-67 in ICC (grade 1); **I:** low proliferation index of Ki-67 in ICC (grade 2); **J:** moderate proliferation index of Ki-67 in ICC (grade 3); **K:** high proliferation index of Ki-67 in ICC (grade 4); **L:** no immunoreactivity of Ki-67 in adjacent tissues (grade 1); **M:** the percentages of Ki-67 in ICC tissues and nonmalignant tissues.

Table 3
Multivariate analysis of recurrence-free survive and overall survival in ICC.

Variables	Recurrence-free survival		*P* value	Overall survival		*P* value
	HR	95% CI		HR	95% CI	
HDAC8 expression	2.203	1.124–4.319	**0.021**	2.107	1.151–3.855	**0.016**
Lymph node metastases	3.058	1.560–5.994	**0.001**	4.366	2.235–8.530	**0.001**

ICC: intrahepatic cholangiocarcinoma; HR: hazard ratio; 95% CI: 95% confidence interval.

HDAC8 was significantly and negatively associated with Ki-67 index ($r = -0.7660$, $P < 0.001$) (Fig. 3).

Low HDAC8 expression suggests poor prognosis of ICC patients

Patients with low HDAC8 expression were proven to have significantly shorter recurrence-free survival ($P = 0.011$) and poorer overall survival ($P = 0.008$) than those with high HDAC8 expression (Fig. 4). The 1- and 3-year recurrence-free survival rates in the low HDAC8 expression group were 29.8% and 11.8%, respectively, while they were 64.1% and 23.9% in the high HDAC8 expression group, respectively (Fig. 4A). Moreover, the 1- and 3-year overall survival rates in the low HDAC8 expression group were lower (37.0% and 5.3%, respectively) than those in the high HDAC8 expression group (69.2% and 25.2%, respectively) (Fig. 4B).

The univariate Cox regression analysis revealed that the expression of HDAC8 levels (hazard ratio (HR) = 2.380, $P = 0.035$) and lymph node metastases (HR = 4.315, $P = 0.002$) were significantly associated with overall survival. Lymph node metastases (HR = 3.495, $P = 0.007$), the levels of CA19-9 (HR = 2.412, $P = 0.023$) and HDAC8 levels (HR = 2.281, $P = 0.046$) were predictors of recurrence-free survival (Table 2). Further analysis of the significant features by the multivariate Cox proportional-hazards model showed that the expression of HDAC8 (HR = 2.107, $P = 0.016$) and lymph node metastases (HR = 4.366, $P = 0.001$) were significantly associated with overall survival. Moreover, HDAC8 expression (HR = 2.203, $P = 0.021$) and lymph node metastases (HR = 3.058, $P = 0.001$) were significantly associated with recurrence-free survival in ICC patients (Table 3).

Discussion

Recent studies have shown that class I HDAC members, including HDAC1, HDAC2, HDAC3 and HDAC8, are associated with

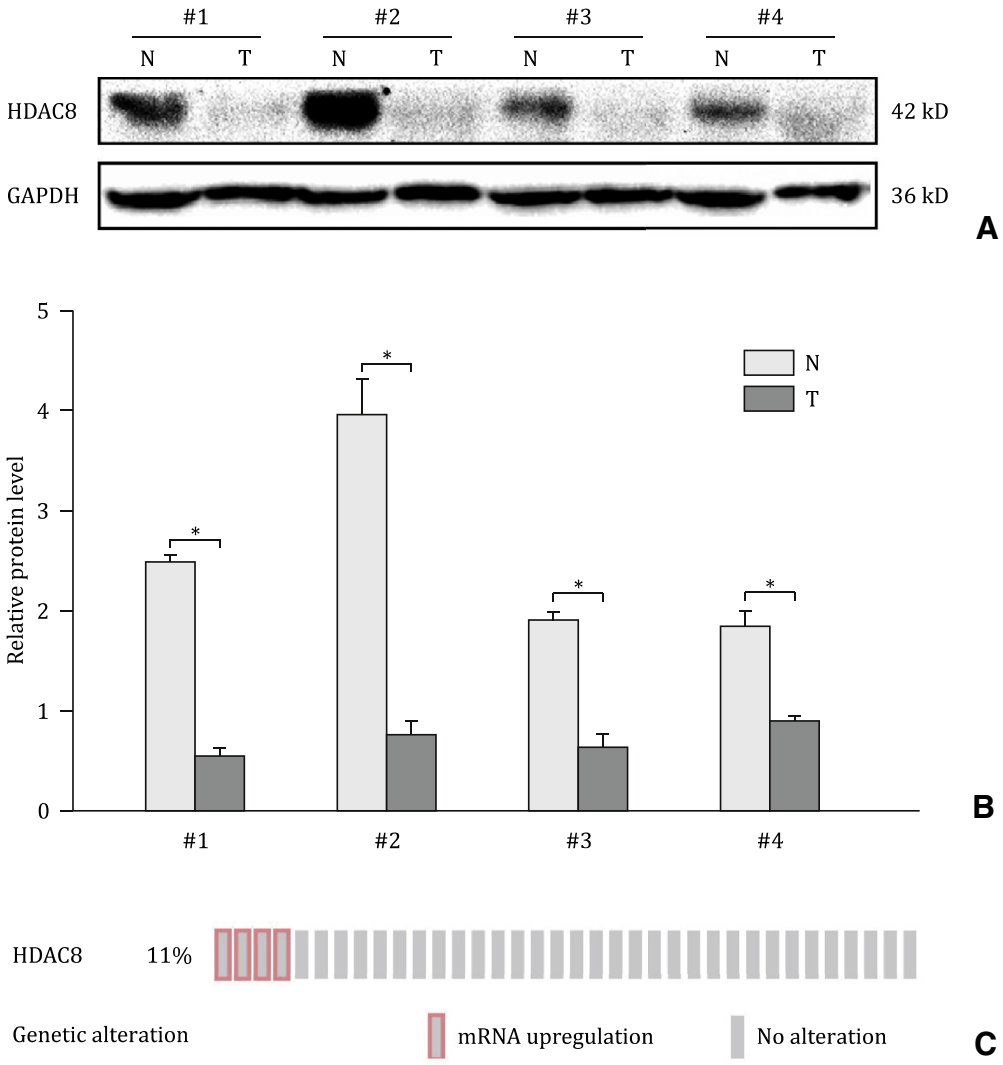

Fig. 2. Western blotting analysis shows that the expression of HDAC8 protein is decreased in ICC tissues compared with the corresponding non-malignant tissues. **A:** HDAC8 expression in ICC tissues and corresponding nonmalignant tissues from 4 different patients. **B:** The relative protein level of HDAC8 normalized by GAPDH. The data are expressed as the mean ± SD of at least three independent experiments. * $P < 0.001$. T: ICC tumor tissue; N: corresponding nonmalignant tissues. **C:** TCGA data showed no alteration in HDAC8 mRNA in 89% (32/36) of ICC tissues.

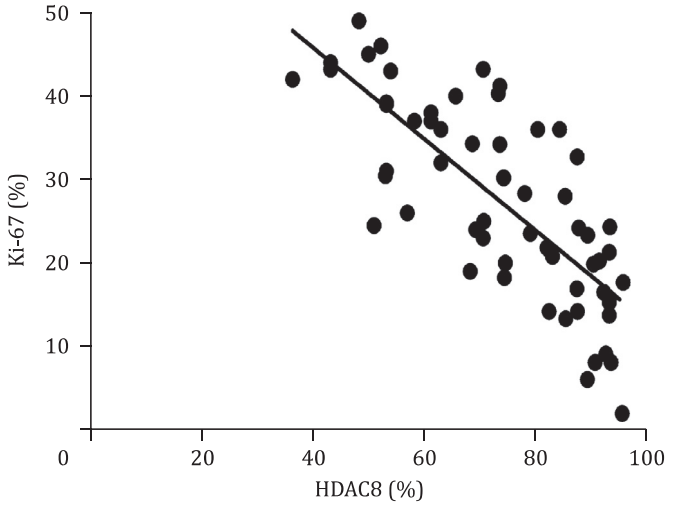

Fig. 3. Pearson correlation analysis showing that the positive cells rate of HDAC8 is significantly and negatively correlated with Ki-67 expression in ICC ($r = -0.7660$, $P < 0.001$).

the clinicopathological characteristics or tumor prognosis in a variety of cancers [31,32]. HDAC1, HDAC2 and HDAC3 play diverse roles and are considered potential biomarkers of ICC [33–35]. As a unique member of the class I HDACs, HDAC8 is associated with several tumors [22–25]. A large number of studies have shown that HDAC8 is highly expressed in most malignancies, including neuroblastoma, lymphomas, HCC, stomach, lung, colon and breast cancer [22,23,36–38]. In contrast to the findings in most cancers showing that HDAC8 overexpression is associated with poor prognosis, our data demonstrated that HDAC8 expression was decreased in most ICC tissues. Most intriguingly, the reduced levels of HDAC8 in ICC tissues were statistically correlated with poorer overall survival and shorter recurrence-free survival.

ICC is not a unique malignancy that exhibits low HDAC8 expression. It has been reported that HDAC8 is absent in prostate cancer cells [37]. In fact, the expression of HDAC8 is still undefined in ICC. Our results showed that HDAC8 expression was downregulated in most ICC tissues. Western blotting analysis validates the results of immunohistochemistry. TCGA data showed that there was no obvious alteration in the mRNA level of HDAC8 in 89% of cases. Using an *in situ* evaluation of HDAC8 protein, we revealed that the levels of HDAC8 were decreased in most ICC tissues. One of the

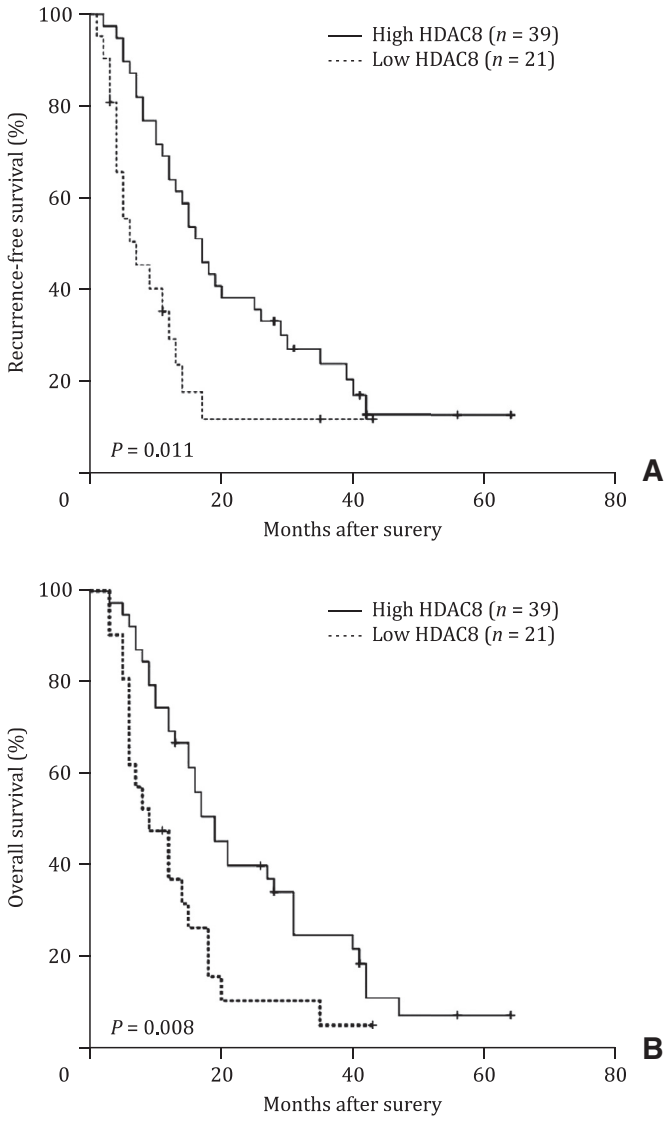

Fig. 4. Kaplan-Meier survival curves by HDAC8 expression in 60 patients with ICC. **A:** recurrence-free survival curves ($P=0.011$); **B:** overall survival curves ($P=0.008$).

reasons is that the mRNA level does not always coincide with the protein level due to posttranscriptional modulation or posttranslational modulation, which has been proven in colorectal tumors showing that amplification or elevation of mRNAs does not always result in the amplification or elevation of protein levels [39].

One of the essential characteristics of malignant cells is their unlimited proliferative capacity. Mutation of HDAC8 can result in abnormal proliferation in several tumors. Loss of HDAC8 activity impairs sister chromatid separation by increasing SMC3 acetylation [18]. HDAC8 promotes abnormal hematopoietic cell proliferation by linking with the inv(16) fusion protein and other co-repressors to repress acute myeloid leukemia-1-regulated genes [26]. In T-cell lymphoma/leukemia, the reduction of HDAC8 suppressed tumor cell growth [40]. It is likely that the increase in HDAC8 activity often promotes tumor growth; however, we observed that the expression of HDAC8 was statistically and negatively correlated with the Ki-67 index in ICC lesions, which suggested that HDAC8 might suppress ICC growth. Unexpectedly, we did observe that decreased HDAC8 did not correlate with tumor size but did correlate with lymph node metastases, which suggested that HDAC8 also has a role in tumor invasion and metastasis.

The pathogenesis of cholangiocarcinoma has been well documented. Cholangiocyte primary cilium as sensory organelle may transduce environmental signals into the cell interior, such as changes in bile composition, of which the quantity reduction and the related genes dysregulation lead to proliferation, invasion, and migration of cholangiocarcinoma by activating non-canonical Hedgehog signaling [41]. The genesis and disassembly of cilium is coordinated with progression through cell cycle, and cilium is composed by a microtubule-based core structure called axoneme [42]. Overexpression of HDAC6 induced by dysregulations of miR-433 and miR-22, which effects on primary cilium, leads to overgrowth and invasion of cholangiocarcinoma [43,44]. HDAC6 is mainly expressed in the cytoplasm, and is activated by HEF1-dependent Aurora A to promote cilium disassembly [45]. Distinct from the class II member HDAC6, HDAC8 is one of class I HDACs and is localized predominantly in the nucleus of ICC cells. Serving as a critical epigenetic regulator, HDAC8 has complicated roles in diverse biological processes. HDAC8 is associated with some special nonhistone proteins, such as cohesin, through which HDAC8 participates in diverse processes, such as sister chromatid separation, and microtubule integrity regulation [18,23].

In conclusion, our findings suggest that low HDAC8 expression promotes lymph node metastases and is significantly associated with worse prognosis in ICC patients. Further studies should be performed to elucidate the molecular effects of HDAC8 in ICC carcinogenesis.

Contributors

BH and SYJ designed the research. ZYJ, XQ, and WZR performed the research. BH and SYJ analyzed the pathological characteristics. SMY and CYW contributed analytic tools. CXY analyzed the data. All authors have read and approved the final manuscript. SYJ is the guarantor.

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Ethical approval

This study was approved by the Ethics Committee of West China Hospital, Sichuan University, Chengdu, China.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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