Original Article
Sirtuin-4 (SIRT4) is downregulated in hepatocellular carcinoma and associated with clinical stage

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Abstract: Background: Several members of the SIRT family (SIRT1-7), a highly conserved family of NAD+-dependent enzymes, play an important role in tumor formation. Recently, several studies have suggested that SIRT4 can both regulate glutamine metabolism and suppress tumor formation. However, our understanding of SIRT4 expression and its association with clinicopathological parameters remains poor. Methods: We evaluated SIRT4 protein expression in hepatocellular carcinoma and corresponding normal liver tissue by immunohistochemistry using a tissue microarray that included 76 hepatocellular carcinoma patients. We also determined the association between SIRT4 expression levels and selected clinicopathological parameters in hepatocellular carcinoma. Results: We found that SIRT4 expression in hepatocellular carcinoma was significantly lower than in corresponding normal tissue (P=0.003). Additionally, decreased SIRT4 levels correlated with T stage (P=0.002) and UICC stage (P=0.002). Conclusions: Our data indicate that SIRT4 may play an important role in the development and progression of hepatocellular carcinoma. Moreover, SIRT4 may be useful as a diagnostic biomarker or therapeutic target in hepatocellular carcinoma.

Keywords: SIRT4, carcinogenesis, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death worldwide [1]. Its incidence has increased in recent years, and there are approximately 750,000 new cases of liver cancer diagnosed each year. Although the 5-year survival rate has improved to roughly 30% with new advances in detection and therapy, this number is still extremely low [2]. The etiology and pathogenesis of liver cancer is quite complex, with several environmental, genetic, and epigenetic risk factors contributing to the disease. Over the last several decades, many key genes and signaling pathways have been implicated in HCC development, including CTNNB1, RASSF1, SOCS1, epithelial growth factor receptor and (EGFR), Ras, (mammalian target of rapamycin pathway) mTOR, WNT/β-catenin, and TGF-β signaling [3, 4]. Despite the progress that has been made, we still do not completely understand the genetic changes that accompany HCC development.

The SIRT family (SIRT1-7) is a group of NAD+-dependent and ADP-dependent transferases, which have been implicated in several cellular processes, including pressure resistance, genome stability, energy metabolism, aging, and tumorigenesis [5]. SIRT1 is the best studied SIRT family member to date; it has been shown to deacetylate both histones and non-histone substrates, including FOXO, p53, and Ku70 [6, 7]. SIRT4, expressed in mitochondria, possesses NAD+-dependent ADP transferase activity, and it catalyzes the transfer of ADP to target proteins, such as GDH [8]. SIRT4 regulates cellular metabolism, including insulin secretion and fatty acid oxidation [8-11]. Recent studies have suggested that SIRT4 elicits tumor suppressor function by inhibiting glutamine metabolism [11, 12]. One group reported that mRNA expression of SIRT4 is decreased in several
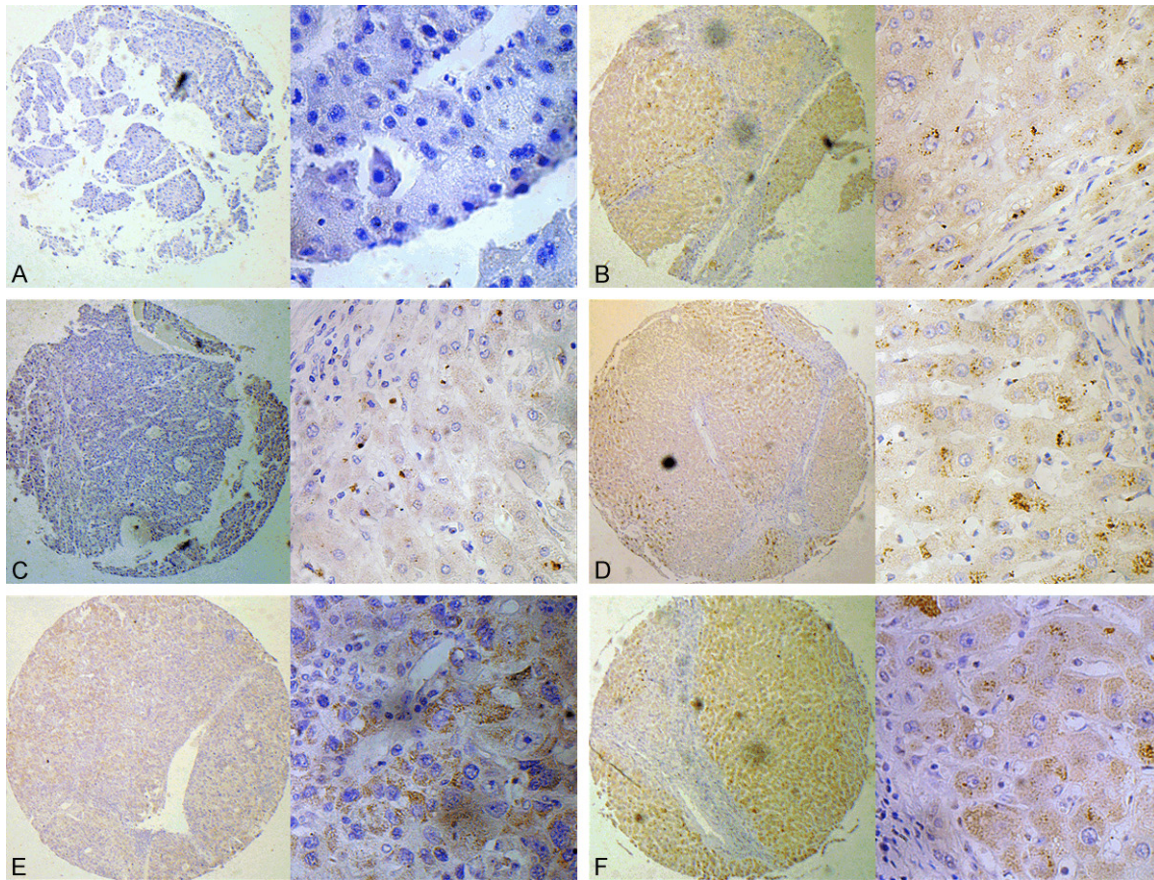
Figure 1. Representative immunohistochemical staining of SIRT4 in human hepatocellular carcinoma tissues. SIRT4 was expressed in the cytoplasm, and was significantly lower in tumor tissues as compared with adjacent normal liver tissue. The micrographs show negative (A), low (C), and high (E) expression of SIRT4 in hepatocellular carcinoma tissues. The relevant expression of SIRT4 in corresponding adjacent normal liver tissues in cases showing (A, C, and E) are shown in (B, D, and F), respectively (Magnification: left panel ×50, right panel ×400).

cancers, including gastric cancer, colon cancer, breast cancer, and thyroid cancer [12]. Earlier work from our group supports these claims; we found decreased SIRT4 protein levels in human gastric cancer tissues that correlated with the pathological differentiation of tumors [13]. Another group reported that low levels of SIRT4 are associated with worse prognosis in colon cancer [14]. Despite these interesting findings, studies examining the precise relationship between SIRT4 and clinicopathological features in human cancers are rare.

In this study, we perform immunohistochemistry to examine the relationship between SIRT4 and liver cancer. We analyzed SIRT4 expression using a tissue microarray containing 76 cases of human hepatocellular carcinoma and normal liver tissues. The expression of SIRT4 in human liver cancer tissues was significantly less than

in normal tissues, and SIRT4 expression significantly correlated with T staging and UICC staging of HCC. Our data suggest that SIRT4 may play an important role in the development of liver cancer.

Materials and methods

Tissue microarray

Tissue microarray was obtained from a commercial chip company (Superchip Inc., Shanghai, China). The chip contained 76 patient samples, including both hepatocellular carcinoma and matched normal liver tissue for each point. In total, there were 152 points on each tissue microarray. The diameter of tissue pieces on the tissue microarray was 1.5 mm, and all points were overlaid with paraffin wax. Of the 76 cases, there were 66 males and 10

Table 1. SIRT4 protein expression in hepatocellular carcinoma and adjacent normal liver tissues

		SIRT4 expression		X^2	P-value[a]
	All cases	Low (%)	High (%)		
Tissue type				43.182	**0.000**
Normal	76	24 (31.6%)	52 (68.4%)		
Cancer	76	64 (84.2%)	12 (15.8%)		

Bold values are statistically significant (P<0.05). [a]Chi-square test.

Table 2. Correlation between the clinicopathologic variables and SIRT4 expression in hepatocellular carcinoma

Clinicopathologic parameters	SIRT4 expression			X^2	P-value[a]
	All cases	Low	High		
Age (years)				1.710	0.222
≤55	44	35	9		
>55	32	29	3		
Gender				0.290	0.694
Male	66	55	11		
Female	10	9	1		
Tumor size (cm)				2.121	0.200
≤5	30	23	7		
>5	46	41	5		
Differentiation				1.249	0.344
Well-Moderate	46	37	9		
Poor	30	27	3		
Stage (T)				12.925	**0.002**
T1	10	6	4		
T2	25	21	4		
T3	36	32	4		
T4	5	5	0		
Stage (N)				1.004	0.587
N0	71	59	12		
N1	5	5	0		
Stage (M)				1.004	0.587
M0	71	59	12		
M1	5	5	0		
UICC stage				12.771	**0.002**
I	10	6	4		
II	25	21	4		
III	35	31	4		
IV	6	6	0		

Bold values are statistically significant (P<0.05). [a]Chi-square test.

therapy prior to surgery. All operations were performed between August and November 2006, with a follow-up period of 4-7 years, which lasted until September 2013. The total survival time was defined as the time of death resulting from a radical operation. Clinical pathology parameters included age, gender, tumor location, tumor size, pathology grade, depth of tumor invasion, lymph node status, UICC staging, and total survival time after surgery.

Immunohistochemistry

Chips were baked in a hot oven incubator for two hours and then placed in xylene for 2×5 min incubations to deparaffinize the specimen. Chips were then transferred to 100%, 100%, 95%, 80%, and 70% successive ethanol washes every 5 min to rehydrate the specimen. Antigen retrieval was performed in a pressure cooker with Tris-EDTA buffer (10 mM Tris-Base, 1 mM EDTA solution, and 0.05% Tween 20, pH 9.0). The chip was then incubated in 0.3% H_2O_2 in TBS for 15 min to suppress endogenous peroxidases. The chip was incubated with an affinity-isolated polyclonal rabbit antibody against SIRT4 (HPA029692, Sigma, USA) at 4°C overnight. Secondary antibody was applied using the GTVision Kit (Gene Tech Inc., Shanghai, China). The chip was stained with diaminobenzidine (DAB) and then counterstained with hematoxylin. The chip was then dehydrated and sealed with coverslips according to standard procedures. Tissue treated with antibody dilution solution was used as a negative control.

Slides were examined by light microscopy by two independent pathologists who were blinded to patient information. Every tissue point was evaluated by both staining intensity (0, no staining; 1, weak staining; 2, strong staining) and staining area (0, <5%; 1, 5%-25%; 2, 25%-50%; 3, 50%-75%; 4, >75%) [15]. The final staining score was obtained by multiplying the staining intensity score by the staining area score. The tissue points were divided into two groups based on the final staining score: low, 0-4; high, 5-8. If the evaluation of the staining was inconsistent, it was

females, with an age-range of 25-73 years (mean, 53.47 ± 10.05 years). None of the patients had received chemotherapy or radio-

Table 3. Univariate analysis of SIRT4 expression and clinicopathologic variables in 76 patients with hepatocellular carcinoma

Variable	All cases	Overall survival (months)		P-value[a]
		Mean	Median	
Age (years)				0.989
≤55	44	36.5	25.0	
>55	32	38.1	29.0	
Gender				0.258
Male	66	39.6	29.0	
Female	10	26.5	16.0	
Tumor size (cm)				**0.031**
≤5	30	48.5	57.0	
>5	46	31.5	17.0	
Differentiation				0.152
Well-Moderate	46	41.6	29.0	
Poor	30	31.9	25.0	
T stage				**0.001**
T1-T2	35	50.1	69.0	
T3-T4	41	28.3	15.0	
N stage				0.408
N0	71	38.8	28.0	
N1-N2	5	28.2	29.0	
M stage				0.408
M0	71	38.8	28.0	
M1	5	28.2	29.0	
UICC stage				**0.001**
I-II	35	49.3	69.0	
III-IV	41	27.4	16.0	
SIRT4 expression				0.940
Low	64	32.32	28.5	
High	12	32.32	29	

Bold values are statistically significant ($P<0.05$). NR, not reached. [a]log-rank test.

reevaluated by the same two pathologists using a multi-headed microscope until all pathologists arrived at a consistent or consensus conclusion.

Statistics

Statistical analysis was performed using software package SPSS version 20.0 (SPSS, Inc.). A paired t test was used to analyze the final score of the tumor and non-tumor tissues. Chi-squared and Fisher's exact tests were used to analyze the relationship between SIRT4 expression and clinicopathological parameters. Kaplan-Meier analysis (log-rank test) was per-

formed for single factor analysis. Cox proportional hazards regression model was used to identify the independent prognostic factors. A *p*-value of <0.05 (two-tailed) was considered statistically significant.

Results

SIRT4 is significantly down-regulated in hepatocellular carcinoma

We performed immunohistochemistry to examine SIRT4 expression in hepatocellular carcinoma and normal liver tissues. SIRT4 was predominantly expressed in the cytoplasm, which is consistent with previously published work [8, 11, 13, 14]. Importantly, the expression of SIRT4 was significantly lower in hepatocellular adenocarcinoma compared to adjacent normal liver tissues (**Figure 1**). We next divided the samples into two groups, defined as low and high, based on the final staining scores. We found that 15.8% (12/76) of hepatocellular adenocarcinoma tissues were scored as SIRT4 high, and 84.2% (64/76) were scored as low. This is in contrast to normal liver tissue, which scored 68.4% (52/76) high and 31.6% (24/76) low (**Table 1**).

SIRT4 expression correlates with several clinicopathological features

To explore the clinical importance of SIRT4 expression in hepatocellular adenocarcinoma, we analyzed the relationship between SIRT4 expression and several clinicopathological features. We found significant relationships between SIRT4 expression and both T stage and UICC stage. Patients that expressed low SIRT4 levels more consistently displayed later T staging later UICC staging. However, we did not find any significant relationships between SIRT4 expression and other parameters, including age, gender, tumor size, pathological differentiation, and vascular invasion (P>0.05). Relationships between SIRT4 and clinicopathological features are summarized in (**Table 2**).

We further analyzed the relationship between SIRT4 expression and prognosis of patients with hepatocellular adenocarcinoma after surgery. We first carried out a single factor analysis. We found that tumor size, T staging, and UICC staging all correlate with total survival time after liver cancer surgery (**Table 3**). How-

Table 4. Cox multivariate analyses of prognostic factors on overall survival

Variables	HR	95% CI	*P*-value[a]
Tumor size (cm) (≤5 versus >5)			n.s.
T stage (T1-T2 versus T3-T4)	2.543	1.434-4.511	**0.001**
UICC stage (I/II versus III/IV)			n.s.
SIRT4 expression (Low versus High)			n.s.

Bold values are statistically significant (*P*<0.05). HR, hazard ratio; CI, confidence interval; n.s., no significance. [a]Forward: LR method.

ever, we did not find any correlation between SIRT4 expression and overall survival (P=0.268) in patients with hepatocellular carcinoma. We next analyzed independent prognostic factors of overall survival in patients with liver cancer by COX regression analysis. After adjusting for the prognostic factors in univariate analysis, we found a significant correlation between T staging and total survival (HR=2.543, P=0.001; **Table 4**).

Discussion

In this study, we analyzed the expression of SIRT4 in hepatocellular carcinoma and its relationship with several clinicopathological features. SIRT4 expression in tumor tissues was significantly less than in normal adjacent liver tissue. We also found significant correlation between SIRT4 expression and T staging and UICC staging in hepatocellular carcinoma. Taken together, our results suggest that SIRT4 may play an important role in the development of liver cancer.

Several SIRT family members have been implicated in cancer, with different family members playing different roles in a tumor type-dependent manner [16]. For example, SIRT1 expression is increased in human gastric carcinoma [17], colon cancer [18], prostate cancer [19], and skin cancer [20]; these observations suggest that SIRT1 may promote tumor formation in these tissues. In contrast, however, some studies show that SIRT1 may act as a tumor suppressor in other tissue types. For example, SIRT1 is down-regulated in breast cancer [21] and can inhibit the formation of intestinal tumors in mice of the APC (Min/+) model [22]. In a similar fashion, a SIRT2 expression is decreased in breast cancer [23], glioma [24], and skin cancer [25] but upregulated in acute myeloid leukemia [26] and prostate cancer

[27]. These findings highlight the diverse roles of SIRT family members in tumorigenesis.

Many of the published studies have supported a tumor suppressor role of SIRT4 in cancer. For example, Jeong *et al.* [11] found that SIRT4 can inhibit glutamine metabolism to suppress tumor formation, and that over-expression of SIRT4 inhibits HeLa cell growth. Moreover, compared to wild-type MEFs, SIRT4-depleted MEFs formed larger tumors when transferred into nude mice. In addition, SIRT4 knockout mice develop several spontaneous tumors, including tumors of the lung, liver, breast, and lymphocytes. Csibi *et al.* [12] found that over-expression of SIRT4 inhibited growth of both the human colon cancer cellline DLD-1 and the human prostate cancer cell-line DU145. Additionally, SIRT4 delayed tumor development in the TSC2-/- (tuberous sclerosis comlex2) MEF xenograft model. Jeong *et al.* [28] found that SIRT4 can suppress Myc-induced B lymphoma cell growth by inhibiting glutamine metabolism. Csibi *et al.* analyzed the online expression microarray database and found that SIRT4 mRNA was down-regulated in human breast cancer, bladder cancer, gastric cancer, colon cancer, thyroid cancer, and ovarian cancer [11, 12]. Here, we find that SIRT4 expression in human HCC is lower than in normal liver. Moreover, we find significant correlation between SIRT4 and tumor staging. And especially, none of Stage IV patients showed high SIRT4 expression (**Table 2**). Our results further support the view that SIRT4 has a role in tumor suppression. Unfortunately, we did not find a statistically significant correlation between SIRT4 and HCC prognosis; which is consistent with the results of Wang JX et al. [15], this could be due to the specificity of SIRT4, or it may be because of the relatively small sample size of this study.

In summary, we show that expression of SIRT4 in HCC is significantly lower than in normal liver tissue. The decrease in SIRT4 expression is significantly correlated with clinical stage of liver cancer. Our results suggest that SIRT4 may play an important role in the development of liver cancer, and it is a potential diagnostic and therapeutic target for HCC.

Disclosure of conflict of interest

None.

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