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Original Article

SIRT4 protein levels are downregulated in kidney renal clear cell carcinoma

Zhouxun Chen[1], Tingting Lei[1], Linfeng Zheng[2], Guoyu Huang[1]

[1]Department of Gastrointestinal Surgery, The First Affiliated Hospital, Wenzhou Medical University, Wenzhou, China; [2]Department of Radiology, Shanghai General Hospital, Shanghai Jiao Tong University, Shanghai, China

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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 may also function as both a tumor oncogene and a tumor suppressor gene. However, no studies have assessed its clinical significance in kidney renal clear cell carcinoma (KIRC). Methods: We investigated SIRT4 protein levels in neoplastic tissues from KIRC patients and its possible association with selected clinicopathological parameters by immunohistochemical staining of a tissue microarray that included 122 KIRC patient tissue samples. Results: SIRT4 protein levels in KIRC were markedly lower than their non-neoplastic tissue counterparts ($P<0.001$). The average survival time of patients with low SIRT4 expression of KIRC was lower than that of patients with high SIRT4 expression, especially in patients older than 60 years and with pathological grade III-IV. Conclusions: Our results indicate that SIRT4 may play a role in the development of KIRC.

Keywords: SIRT4, carcinogenesis, kidney renal clear cell carcinoma

Introduction

Renal cell carcinoma (RCC), the seventh most common tumor worldwide, is associated withmore than 130,000 deaths per year [1]. Kidney renal clear cell carcinoma (KIRC), which represents approximately 80% of allprimary kidney malignancies, is the predominant histologic subtype of RCC [2]. The etiology and pathogenesis of KIRC is highly complex and involves many risk factors and a variety of genetic and epigenetic alterations. Over the past few decades, many key genes and signaling pathways, such as VHL, VEGF, TGF-β, PDGF-B, HIF-α and MET, were found to play key roles in the pathogenesis of KIRC [3].

The SIRT family (SIRT1-7) is a group of NAD^+-dependent deacetylases and ADP-ribosyl transferases that regulate pressure resistance, genomic stability, energy metabolism and aging [4]. In particular, SIRT4 is a mitochondrial localized NAD^+-dependent ADP-ribosyltransferase that catalyzes the transfer of ADP ribosyl toglutamate dehydrogenase (GDH) [5]. Additionally, SIRT4 regulates insulin secretion, fatty acid oxidation, and metabolic function [5-8]. Recent studies indicate that SIRT4 exerts tumor suppressor capabilities by regulating glutamine metabolism [8, 9]. Several studies have also found that SIRT4 expression is downregulated in gastric and colon cancer tissues and is associated with pathological grade and otherclinicopathologic parameters [10-12]. So far, no studies have reported the relationship between SIRT4 expression and the clinical pathological parameters of KIRC. By employing high-throughput tissue microarray and immunohistochemistry, we investigated the expression of SIRT4 protein in neoplastic tissues from KIRC patients and analyzed the relationship between SIRT4 protein and the clinically relevant pathological parameters in KIRC.

Materials and methods

This study was approved by the First Affiliated Hospital of Wenzhou Medical University (Wen Zhou, China) and was conducted in accordance with the principles of the Declaration of Helsinki.

Table 1. Correlation between SIRT4 expression and clinicopathological variables in KIRC

Clinicopathological parameters	SIRT4 expression			X^2	*P*-value[a]
	All cases	Low	High		
Age (years)				0.268	0.604
≤60	76	46	30		
>60	46	30	16		
Gender				2.560	0.110
Male	92	61	31		
Female	30	15	15		
Tumor size (cm)				0.106	0.745
≤5	64	39	25		
>5	58	37	21		
Differentiation				2.708	0.100
I-II	85	57	28		
III-IV	37	19	18		
Stage (T)				0.430	0.512
T1a	59	35	24		
T1b-T3	63	41	22		
Stage (N)				0.025	0.847
N0	119	74	45		
N1	3	2	1		
AJCC stage				0.001	0.982
I	98	61	37		
II-IV	24	15	9		

[a]Chi-square test.

Patients and tissue samples

For this study, 122 individual KIRC patient tissue samples were procured from February 2008 to March 2010, with a follow-up ranging from 5.5-7.5 years beginning in August 2015. The patients' ages ranged from 24 to 83 years with a mean of 57 years. The patients did not receive preoperative chemotherapy or radiotherapy before surgery. Overall survival was determined at the time of any radical surgery or death. The clinicopathologic parameters included the following: patient age, gender, tumor size, pathological grade, depth of tumor invasion, lymph node status and the American Joint Committee on Cancer (AJCC, 7th edition) staging information.The major clinicopathological parameters are shown in **Table 1**.

Tissue gene array chips were commercially obtained (Superchip Inc., Shanghai, China). There were 122 patient samples, and 16 cases contained both KIRC and the corresponding adjacent non-neoplastic tissues specimen for eachpoint. Thus, there were 138 points on one tissue microarray. The diameter of tissue pieces on the tissue microarray was 1.5 mm, andall points were overlaid with paraffin wax.

Immunohistochemistry

The tissue microarray was first prepared by incubationin a 60°C oven incubator for 2 hrs followed by 2 incubations in xylene for 5 min at room temperature to deparaffinize the specimen. The tissue microarray was then transferred to successively graded concentrations of ethanol washes at 100%, 100%, 95%, 85%, and 70% every 5 min to rehydrate the specimen. Antigen retrieval was performed in a pressure cooker with citrate buffer (10 mM citrate and 0.05% Tween 20, pH 6.0) at 170 kPa at 120°C for 5 minutes. The microarray chip was then incubated in 0.3% H_2O_2 in Tris-HCl buffer for 15 min at room temperature to suppress endogenous peroxidase activity. The tissue microarray was then incubated with polyclonal rabbit anti-SIRT4 (HPA029692, 1:400, Sigma, USA) at 4°C overnight. A secondary antibody was applied using the GTVision Kit (Gene Tech Inc., Shanghai, China). The microarray chip section was then stained with diaminobenzidine (DAB) and counterstained with hematoxylin. Next, the chip was dehydrated and sealed with a coverslip. Tissue that was treated with dilution solution alone (no antibody) served as a negative control.

Two pathologists performed blinded analysis of the SIRT4 immunostaining intensity under a light microscope. Each tissue point was assigned a score based on the staining intensity multiplied by the area of the stain [13]. The staining intensity was divided into four categories: 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining. Staining area assessment was as follows: 0 = 5% or none of the cells were stained, 1 = 5-25% of the cells stained positive, 2 = 26-50% of the cells stained positive; 3 = 51-75% of the cells stained positive; and 4 = more than 75% of the

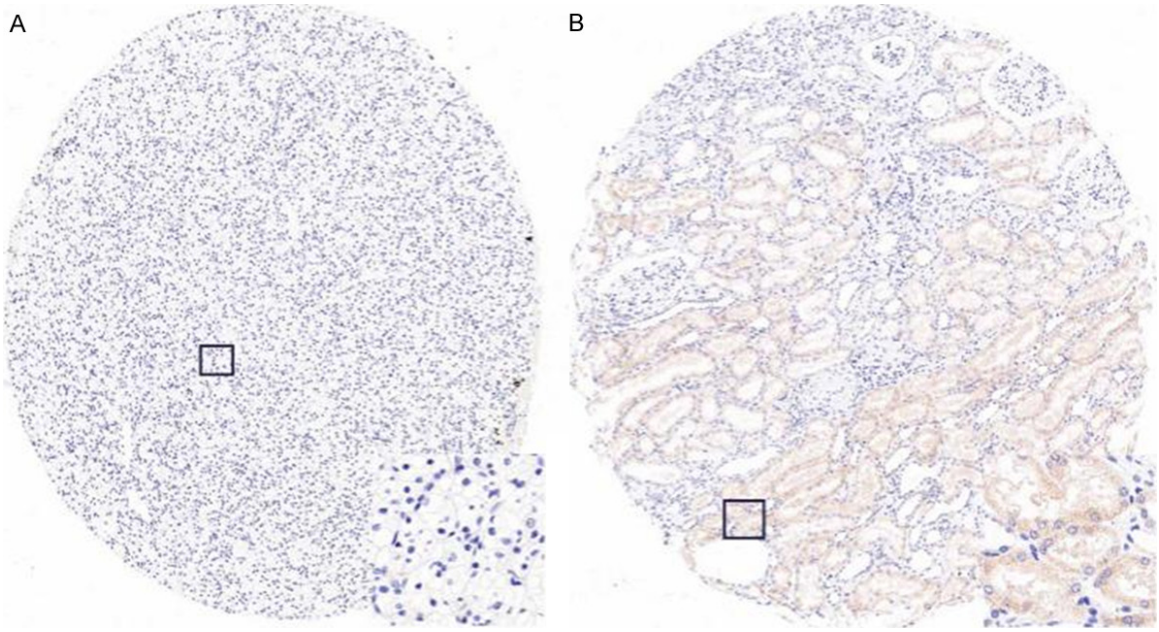

Figure 1. Representative immunohistochemical staining of SIRT4 in tumor cells. SIRT4 was localized to the cytoplasm and was expressed at lower levels in tumor tissues as compared with adjacent non-neoplastic kidney tissues. A. The micrographs showed weak staining of SIRT4 in the KIRC tissues. B. Relevant expression of SIRT4 in corresponding adjacent non-neoplastic kidney tissues. (Magnification: 100× and 400×).

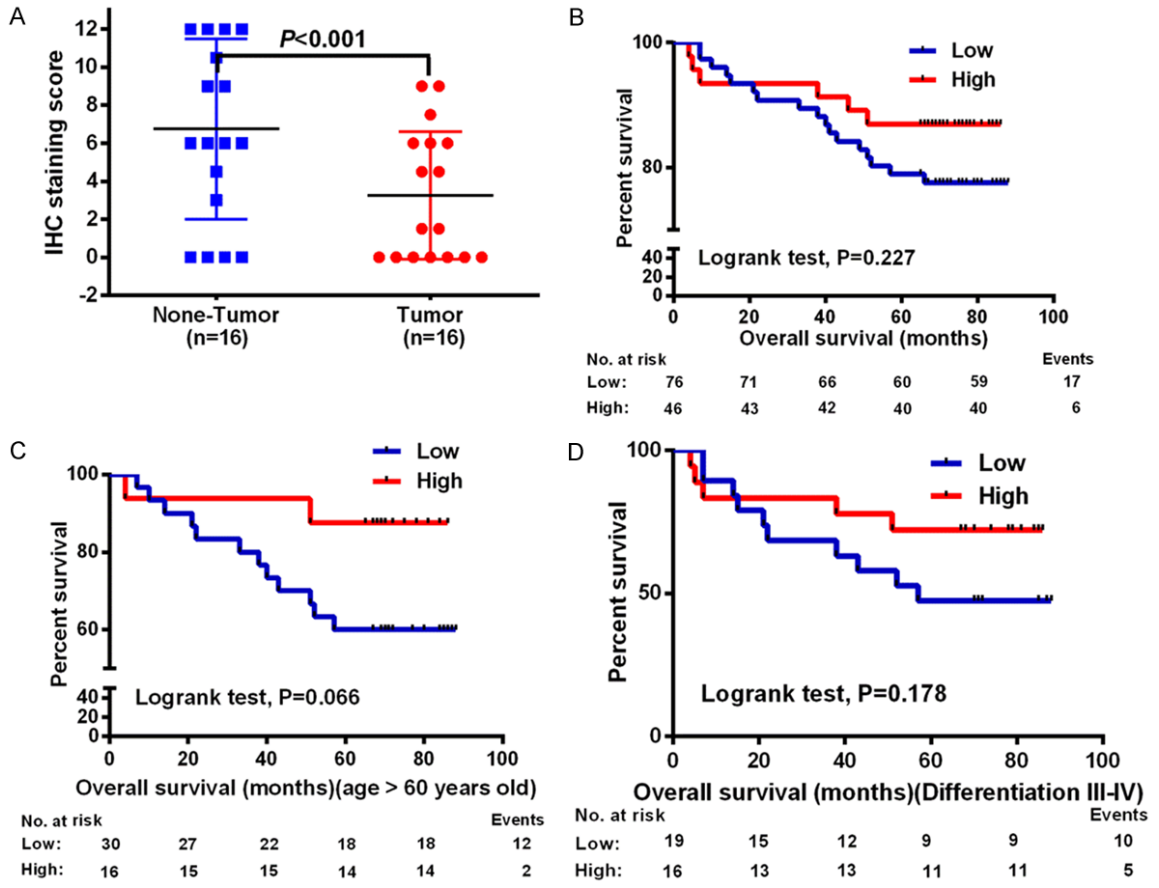

Figure 2. A. SIRT4 protein levels measured in 16 KIRC tissues and paired adjacent normal esophageal tissues by tissue microarray. SIRT4 protein levels were lower in tumor tissues compared with adjacent non-neoplastic kidney tissues (P<0.001). The boxes represent the interquartile range; whiskers represent the 5th-95th percentile range; and bars represent the median. B. The average survival time of patients with KIRC with low SIRT4 levels was lower than that of patients with high SIRT4 levels (P = 0.227). C. The average survival time of KIRC patients (age>60 years old) with low SIRT4 levels was lower than that of patients with high SIRT4 levels (P = 0.066). D. The average survival time of KIRC patients (Differentiation III-IV) with low SIRT4 levels was lower than that of patients with high SIRT4 levels (P = 0.178).

Table 2. Univariate analysis of SIRT4 expression and clinicopathological variables in 122 patients with KIRC

Variable	All cases	Overall survival (months)		*P*-value[a]
		Mean	Median	
Age (years)				**0.011**
≤60	76	81.2	NR	
>60	46	70.8	NR	
Gender				0.166
Male	92	80.9	NR	
Female	30	75.8	NR	
Tumor size (cm)				**0.003**
≤5	64	84.1	NR	
>5	58	62.6	NR	
Differentiation				**0.001**
I-II	85	83.7	NR	
III-IV	37	62.5	NR	
T stage				**0.000**
T1a	59	85.1	NR	
T1b-T3	63	70.0	NR	
N stage				**0.000**
N0	119	85.1	NR	
N1-N3	3	14.0	15.0	
AJCC stage				**0.000**
I-II	98	83.4	NR	
III-IV	24	52.5	46.0	
SIRT4 expression				0.227
High	46	78.1	NR	
Low	76	77.8	NR	

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

cells stained positive. Finally, the degree of staining was divided into two categories: 0-5 = low expression; and 6-12 = high expression. Both pathologists formulated a consensus opinion when their evaluation of the staining pattern did not agree.

Statistical analysis

Statistical analysis was performed using the SPSS software package version 20.0 (SPSS, Inc., IBM, USA). A paired Student's t-test was used to analyze the final score of the tumor and non-tumor tissues. Chi-squared analysis was used to analyze the relationships between SIRT4 expression and the clinicopathological parameters. The Kaplan-Meier method (the log-rank test) was used for single-factor analysis. The Cox proportional hazards regression model was used to identify the independent prognostic factors. *P*<0.05 (two-tailed) was considered statistically significant.

Results

SIRT4 expression in KIRC and adjacent non-neoplastic tissues

SIRT4 was predominantly expressed in the cytoplasm (**Figure 1**). Importantly, the staining intensity of SIRT4 was lower in neoplastic samples from KIRC patients as compared to adjacent non-neoplastic endometrial tissues (**Figure 2A**).

Relationship between SIRT4 levels and clinicopathological parameters in KIRC patients

Associations between SIRT4 levels and clinicopathological features were evaluated using immunohistochemistry (**Table 1**). We did not find any significant associations between SIRT4 levels and parameters including age, gender, pathological grade, tumor size, and T, N and AJCC staging (P >0.05).

Association between SIRT4 levels and total survival time of patients with KIRC after operation

The Kaplan-Meier analysis and log-rank test were used to investigate the prognostic value of SIRT4 levels for patient survival. In univariate analysis, we found that age, tumor size, pathological grade, and T, N and AJCC staging were associated with overall survival in patients with KIRC (**Table 2**). However, we did not find a correlation between the expression of SIRT4 and the total survival time of patients with KIRC (P =

Table 3. Cox multivariate analysis of prognostic factors on overall survival

Variables	HR	95% CI	*P*-value[a]
Age (years) (≤60 versus >60)	1.432	0.586-3.500	0.431
Gender (male versus female)	2.057	0.580-7.299	0.264
Tumor size (cm) (≤5 versus >5)	0.806	0.175-3.710	0.782
Differentiation (I-II versus III-IV)	2.632	0.997-6.947	0.051
T stage (T1a versus T1b-T3)	2.055	0.296-14.284	0.467
N stage (N0 versus N1)	5.692	1.320-24.542	**0.020**
AJCC stage (I versus II-IV)	3.510	1.157-10.647	**0.027**
SIRT4 expression (Low versus High)	0.741	0.280-1.966	0.548

Bold values are statistically significant ($P<0.05$). HR, hazard ratio; CI, confidence interval; [a]Forward method.

0.227). Although not statistically significant, the average survival time of patients with low SIRT4 levels in KIRC tissues was lower than that of patients with high levels (**Figure 2B**). Nevertheless, in KIRC patients older than 60 years old and with pathological grade III-IV, the overall survival time was lower in patients with low SIRT4 expression compared with patients with high SIRT4 levels (**Figure 2C** and **2D**).

COX regression analysis was further used to analyze the independent prognostic factors for overall survival time of patients with KIRC. After adjusting the prognostic factors in univariate analysis, there was a significant correlation between N staging and AJCC staging and total survival (**Table 3**).

Discussion

Based on studies published in the literature, multiple SIRT family members play a variety of roles in different tumor types. These various roles may depend on the specific tissue and tumor type [14]. For example, the presence of SIRT1 in stomach [15], colon [16], prostate [17], skin [18] cancers, as well as several other tumors, suggests that SIRT1 might promote tumor development in these cancers. Conversely, other studies have found reduced SIRT1 expression in human breast cancer [19]. Moreover, SIRT1 expression in the mouse $APC^{min/+}$ model inhibits the formation of intestinal tumors [20]. In addition, this particular observation is similar to that found for SIRT2, which was down-regulated in human breast [21], glioma [22] and skin cancers [23]. However, SIRT2 expression was enhanced in acute myeloid leukemia [24] and prostate cancer [25]. Thus, we cannot easily extrapolate the observations and conclusions made for one tumor type to that of another-tumor type.

At present, some studies have found that SIRT4 also plays a role in cancer. For example, Jeong et al. [8] found SIRT4 can suppress tumor formation by inhibiting glutamine metabolism, overexpression of SIRT4 can inhibit the growth of HeLa cells, SIRT4 knockout MEF cells formed larger tumors in nude mice, and SIRT4 knockout mice spontaneously generated lung cancer, liver cancer, breast cancer and lymphoma. Csibi et al. [9] also found that overexpression of SIRT4 can inhibit the growth of the human colon cancer cell line DLD-1 and human prostate cancer cell line DU145. In further support of these findings, RT-PCR analysis of mRNA extracted from human tissue demonstrated that the mRNA level of SIRT4 in colon [13], breast [26], endometrial [27] and oesophageal cancers [28] is reduced. In addition, decreased SIRT4 protein levels in gastric, colon, liver and oesophageal cancer tissues are associated withpoor pathological grading and other clinicopathological parameters [10, 11, 13, 29]. More specifically, reduced SIRT4 protein levels correlate with poor prognosis in colon and oesophageal cancer [11, 13, 28]. Recently, a study found that SIRT4, which was decreased in non-small cell lung cancer (NSCLC), couldinhibit lung cancer cell proliferation, block cell cycle, and repress cell invasion and migration [30]. These studies suggest that SIRT4 may function as a tumour suppressor.

To date, no study has reported the relationship between SIRT4 expression and the clinicopathological parameters of patients with KIRC. In the present study, we analyzed the SIRT4 protein levels in neoplastic tissues from KIRC patients and the relationship between SIRT4 levels and the clinicopathological parameters of patients with KIRC. We found that SIRT4 is significantly downregulated in KIRC as compared with adjacent non-neoplastic tissues. Moreover, the average survival time of patients with low SIRT4 levels in KIRC tissues was lower

than that of patients with high levels, especially in patients older than 60 years andwith pathological grade III-IV. These observations suggest that SIRT4 may have some regulatory role in the development of KIRC.

Current research showed that SIRT4 can inhibit tumor metabolism, especially glutamine metabolism [8, 9, 31], therefore playing the role of a tumor suppressor gene. SIRT4 is thought to be a keeper of cell energy metabolism [31]. Indeed, altered energy metabolism is a key feature of tumors [32]. Tumor cells and normal cells have distinct metabolic patterns. Tumor cells often appear to enhance glucose and glutamine metabolism to meet the energy demand of tumor growth [33, 34]. However, understanding the mechanism of SIRT4 action requires more research. Like other members of the SIRT family, SIRT4 may also have a complex regulatory network and may assume the role of both tumor suppressor gene and oncogene depending on the context. Therefore, we should continue to explore the SIRT4 regulatory network in KIRC tumors in order to facilitate in-depth understanding of its role in cancer.

To the best of our knowledge, our study is the first to analyze the relationship between SIRT4 expression levels and clinicopathological parameters in human KIRC specimens, especially at the protein level. Moreover, for the first time,we found that the average survival time of KIRC patients with low expression of SIRT4 was lower than that of patients with high expression, especially in patients older than 60 years andwith pathological grade III-IV. Although the differences were not statistically significant, our results indicate the necessity for further study on the role of SIRT4 in renal cell carcinoma. Thus, the next step will be to use a larger sample size from various tumor types to further determine the relationship between SIRT4 levels and the prognosis of KIRC patients. This approach will allow us to further study the effect of SIRT4 on the biological behavior of KIRC cells. In summary, our results suggest that SIRT4 may participant in the development of KIRC.

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Disclosure of conflict of interest

None.

Address correspondence to: Tingting Lei and Guoyu Huang, Department of Gastrointestinal Surgery, The First Affiliated Hospital, Wenzhou Medical University, Wenzhou 325000, China. E-mail: leitingting_wz@163.com (TTL); huangguoyu.greg@gmail.com (GYH); Linfeng Zheng, Department of Radiology, Shanghai General Hospital, Shanghai Jiao Tong University, Shanghai 200080, China. E-mail: zhenglinfeng04@aliyun.com

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