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Original Article

The expression and correlation of SIRT1 and Phospho-SIRT1 in colorectal cancer

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Abstract: SIRT1 is the homologue of sir2 in mammals, which is a nicotinamide adenine dinucleotide (NAD^+) dependent histone deacetylase. SIRT1 is involved in many physiological processes, such as metabolism, senescence, inflammatory response, neuroprotection, and tumorigenesis by acetylating histones and multiple transcription factors. However, the exact role of SIRT1 in tumor is still under controversial. Immunohistochemistry and Western blot were performed to investigate the expressions and subcellular localizations of SIRT1 and Phospho-SIRT1 in colorectal cancer tissues and adjacent normal tissues. The relationship between SIRT1 or Phospho-SIRT1 and clinicopathological characteristics was also analyzed. Real-Time PCR was performed to investigate the transcriptional level of SIRT1 mRNA in colorectal cancer tissues and adjacent normal tissues. SIRT1 and Phospho-SIRT1 were both localized in the nucleus. The expressions of SIRT1 and Phospho-SIRT1 were higher in colorectal cancer tissues than normal tissues. SIRT1 expression in cancer tissues was associated with patient age, TNM stage and mutant P53 loss. Phospho-SIRT1 expression in cancer tissues was associated with Ki67. SIRT1 and Phospho-SIRT1 were highly correlated in cancer tissues and normal tissues. The ratios of Phospho-SIRT1 and SIRT1 expression in cancer tissues were higher than normal tissues. SIRT1 mRNA level was no significant difference in cancer tissues and normal tissues. SIRT1 have a dual character in colorectal cancer, and Phospho-SIRT1 may determine the role of SIRT1 in colorectal cancer formation.

Keywords: SIRT1, Phospho-SIRT1, colorectal cancer, Ki67, P53

Introduction

Silent information regulator 2 (Sir2) is an anti-aging gene discovered in budding yeast originally, which encodes a protein with NAD^+ dependent histone deacetylase activity [1]. SIRT1 is one of seven homologs of Sir2 in mammals [2], which involved in cell energy metabolism, proliferation, senescence, multiple inflammatory processes, neuroprotection, tumorigenesis and so on [3, 4]. However, there are many controversies on the role of SIRT1 in tumors [5].

SIRT1 maybe is a tumor promoter. SIRT1 overexpression was observed in prostate cancer [6], colon cancer [7], ovarian cancer [8], breast cancer [9], gastric cancer [10], pancreatic cancer [11], hepatocellular cancer [12], acute myeloid leukemia [13] and diffuses large B cell lymphoma [14], which associated with low grade, advanced TNM stage, lymph node metastasis and short overall survival. SIRT1 represses cellular apoptosis by deacetylating P53 [15], KU70 [16], FOXO [17] and other substrates, and promotes cellular metastasis by deacetylating Cortactin and ZEB1 [18-20]. Researchers reported that shRNA or SIRT1 inhibition could reverse drug resistance [21-23].

SIRT1 maybe is a tumor suppressor. Some studies have found low expression of SIRT1 in colon cancer, breast cancer, hepatocellular cancer, prostate cancer, ovarian cancer and bladder cancer, which associated with short overall survival [24, 25]. SIRT1 manipulates deacetylate of NF-κB [26], β-catenin [27], Survivin [28] and c-Myc [29] to promote cellular apoptosis. In mouse fibroblast cell lines MEFs, SIRT1 activator resveratrol can reduce the inflammatory

response induced by TNF-α [30]. In addition, Wang et al. 2008b observed early mortality and chromatin instability in mutant SIRT1-/- mouse [25].

SIRT1 was originally thought to be a nucleoprotein [31]. More and more studies have found that SIRT1 subcellular localization was diversity-completely localized in the nucleus [9, 10, 12, 24], or completely localized in the cytoplasm [32, 33], or both localized in the nucleus and cytoplasm [8, 18, 34]. It has been demonstrated that SIRT1 contains two nuclear localization signals and two nuclear export signals, and subcellular localization of SIRT1 was due to the nucleocytoplasmic shuttling [32, 35].

SIRT1 is regulated by series of factors at transcription, translation and post-translational modification stages. Studies had pointed out that SIRT1 protein level was not associated with SIRT1 mRNA level, indicating that phosphorylation played an important role in regulating the activity of SIRT1 deacetylation and nucleocytoplasmic shuttling [36, 37]. However, researches on Phospho-SIRT1 in tumors are less common.

In this research, we observed the expressions and subcellular localizations of SIRT1 and Phospho-SIRT1 in colorectal cancer tissues and adjacent normal tissues, and investigated the correlation between the expressions and clinicopathological characteristics. We also observed SIRT1 mRNA in colorectal cancer tissues and adjacent normal tissues to determine the role of phosphorylation.

Materials and methods

Samples collection and reagents

50 cases of colorectal cancer paraffin tissues and 20 cases of adjacent normal paraffin tissues were collected in Liaocheng People's Hospital from January 2012 to December 2013. 20 cases of fresh cancer tissues and adjacent normal tissues were collected immediately after surgery, and stored at -80°C. Adjacent normal tissue refer to the tissue that have more than 5 cm distance from the cancerous tissue, and no pathologically confirmed invasive cancer. All samples have clear diagnosis and complete pathological data. Preoperative chemotherapy was not carried out. Ethics committees have given the approval for the use of all samples.

50 cases of colorectal cancer paraffin tissues were shown as follows: 32 males, 18 females; aged ≥ 60 years 29 cases, 21 cases less than 60 years old; well differentiated type 3 cases, moderate differentiated type 41 cases, poorly differentiated-type 6 cases; I of 13 cases, II of 17 cases, III of 19 cases, IV of 1 cases; lymph node metastasis 19 cases; distant metastasis 1 cases.

Antibodies

Antibodies for immunohistochemistry (IHC): SIRT1 (1104-1, Epitomics, 1:100), Phospho-SIRT1 (pS47) (2381-1, Epitomics, 1:1000), p53 (MAB-0142, maixin, China, 1:150), Ki67 (MAB-0542, maixin, China, 1:200). EliVision™ plus detection Kit (KIT-9903, maixin, China).

Antibodies for Western blot (WB): SIRT1 (1104-1, Epitomics, 1:2000), Phospho-SIRT1 (pS47) (2381-1, Epitomics, 1:2000), β-actin (AA128, Beyotime, China, 1:1000), H3 (AH433, Beyotime, China, 1:1000), goat anti-rabbit HRP-conjugated IgG (H + L) (A0208, Beyotime, China, 1:1000), goat anti-mouse HRP-conjugated IgG (H + L) (A0216, Beyotime, China, 1:1000).

Immunohistochemical analysis

SIRT1, Phospho-SIRT1, p53 and Ki67 were detected according to the procedure of EliVision plus detection Kit. Briefly, 3 μm paraffin slides were heated at 65°C for 30 mins. Antigen retrieval was performed for 2 mins in Pressure Cooker containing sodium citrate buffer after deparaffinization and hydration. Inactivate endogenous peroxidase with 3% hydrogen peroxide for 10 mins and block slides with 5% BSA for 1 hour. Primary antibodies were incubated for 1 hour at room temperature. HRP-conjugated secondary antibodies were applied to each slides and incubated for 30 mins at room temperature.

Evaluation of IHC staining

Two independent pathologists read the slides in case of unknown clinical data. Final score was dependent on both staining density and staining area. The staining density was graded as follows: 0, no staining; 1, weak staining; 2,

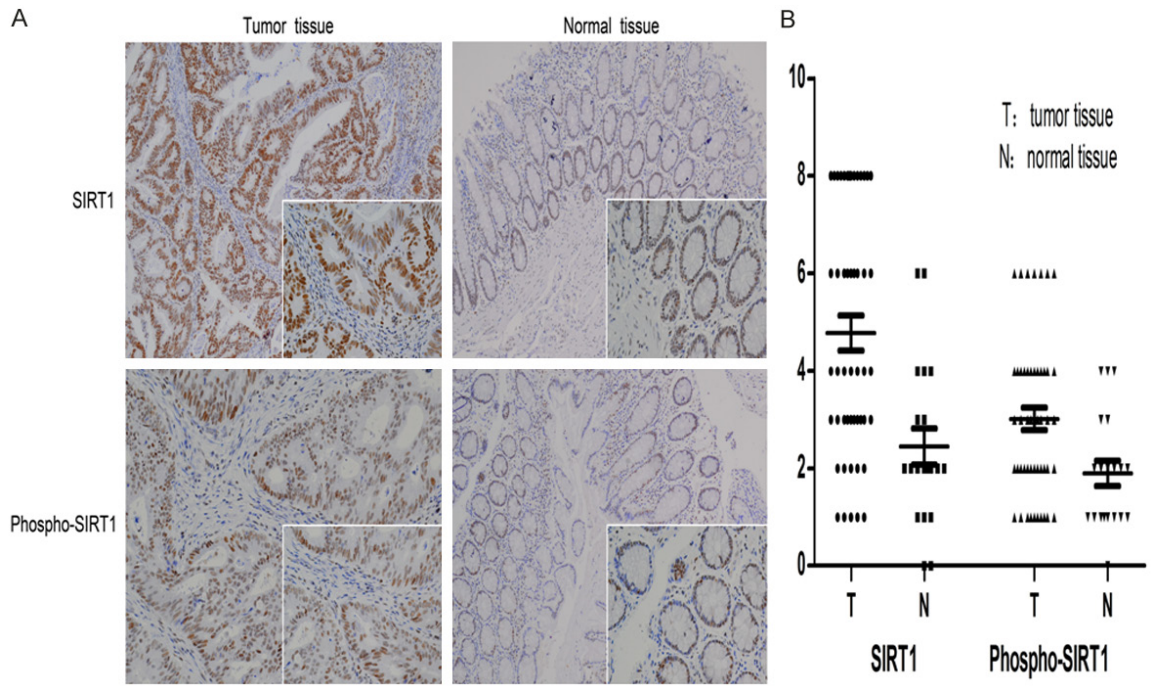

Figure 1. SIRT1 and Phospho-SIRT1 were detected by IHC (100 × and 400 × magnification). Mann-Whitney U test was used to analysis expression difference of SIRT1 or Phospho-SIRT1 in cancer tissues and normal tissues. A. Brown staining was seen localized to the nucleus of cells. SIRT1 expression in cancer tissues and normal tissues were 60% and 25%, respectively, Phospho-SIRT1 expression were 38% and 15%, respectively. B. Final IHC staining score of each slide was scored by multiplying the staining density by the staining area, and staining scores were shown as a scatter plot. The average score of SIRT1 in cancer tissues and normal tissues were 4.780 ± 0.353 and 2.450 ± 0.373, respectively ($P < 0.05$), the average scores of Phospho-SIRT1 were 3.020 ± 0.228 and 1.900 ± 0.261, respectively ($P < 0.05$).

moderate staining; 3, intense staining. Percentage area was graded as follows: 0, < 5%; 1, 5% to 25%; 2, 26% to 50%; 3, 51% to 75%; 4, > 75%. The final score of SIRT1 and Phospho-SIRT1 was scored by multiplying the staining density by the staining area, and positive was regarded if final scores ≥ 4. P53 was considered positive if ≥ 30% tumor cells were stained. Ki67 was considered positive if ≥ 50% tumor cells were stained.

Western blot analysis

Fresh frozen tissues were extracted respectively for whole protein, cytoplasm protein and nucleus protein by RIPA buffer and nucleus-cytoplasm protein extraction kit. Protein concentration was determined by BCA assay and boiled at 100°C for 5 min mixed with loading buffer. Proteins were separated on SDS-PAGE gel and transferred to PVDF membrane. PVDF membrane was blocked by 5% non-fat milk for 1.5 hours at room temperature. Primary antibodies were incubated overnight at 4°C and HRP-conjugated secondary antibodies were incubated for 1h at room temperature. Immunoreactive signals were detected by ECL. Cytoplasm protein were normalized with β-actin, nucleus protein were normalized with H3.

Real Time transcription-polymerase chain reaction (Real-Time PCR)

Total RNA was isolated from colorectal cancer tissues and adjacent normal tissues with Trizol reagent (Beyotime, China). cDNA was synthesized by QuantScript RT Kit (KR103, TIANGEN, China). Prepare the reaction system according to SuperReal PreMix Plus (SYBR Green) (FP205, TIANGEN, China). GAPDH was selected as an internal control gene. The primer sequences were as follows: SIRT1 forward 5'-CCGGATTTGAAGAATGTTGG-3', SIRT1 reverse 5'-ATCTGCTCCTTTGCCACTCT-3', GAPDH forward 5'-GGTATCGTCGAAGGACTCATGAC-3', GAPDH reverse 5'-ATGCCAGTGAGCTTCCCGTTCAGC-3'.

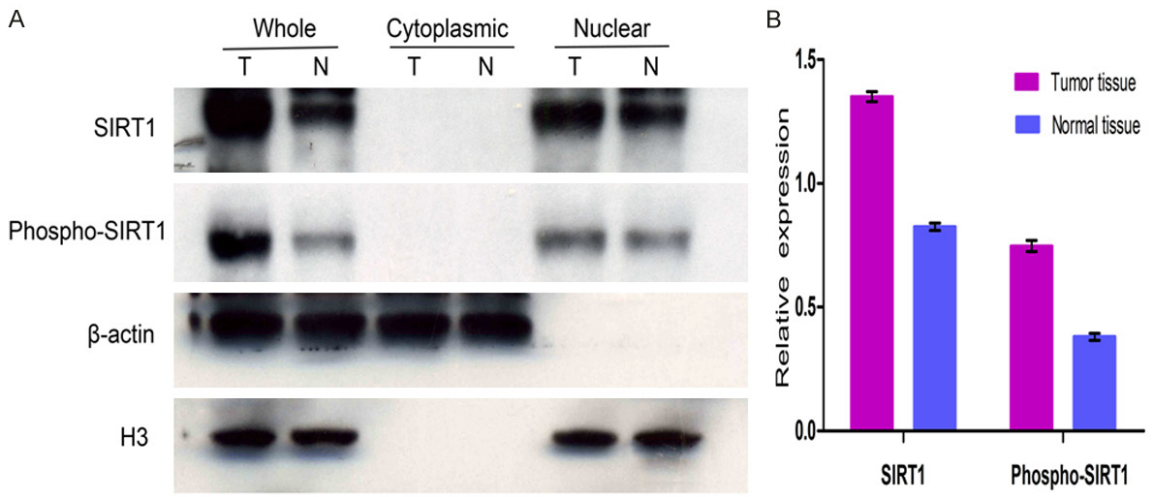

Figure 2. SIRT1 and Phospho-SIRT1 were detected by WB. Mann-Whitney U test was used to analysis expression differences of SIRT1 or Phospho-SIRT1 in cancer tissues and normal tissues. A. Tumor tissues and normal tissues were both extracted for whole protein, cytoplasmic protein and nuclear protein (T = tumor tissue, N = normal tissue). Cytoplasm protein was normalized with β-actin and nucleus protein was normalized with H3. SIRT1 and Phospho-SIRT1 were both localized in the nucleus of cancer tissues and normal tissues. B. Relative expression of SIRT1 and Phospho-SIRT1 in tumor tissues and normal tissues. SIRT1 expression in cancer tissues and normal tissues were 1.351 ± 0.020 and 0.825 ± 0.015, respectively ($P < 0.05$), Phospho-SIRT1 expression were 0.747 ± 0.022 and 0.381 ± 0.0148, respectively ($P < 0.05$).

Statistical analysis

SPSS 19.0 software was used to analysis the data. Mann-Whitney U test was used to analysis expression differences of SIRT1 or Phospho-SIRT1 in cancer tissues and normal tissues. The χ^2 test or Fisher's exact test was used to evaluate the differences between SIRT1 or Phospho-SIRT1 expression and clinicopathological characteristics in colorectal cancer tissues. Spearman correlation analysis was used to examine the expression correlation between Phospho-SIRT1 and SIRT1. SIRT1 mRNA expression differences in cancer tissues and normal tissues were analyzed using the comparative C_T method. All values were expressed as mean ± standard error (Mean ± SE). *P* value < 0.05 was considered statistically significance.

Results

SIRT1 and Phospho-SIRT1 were both localized in the nucleus and expressed in colorectal cancer tissues.

IHC revealed that SIRT1 and Phospho-SIRT1 were both localized in the nucleus of cancer tissues and normal tissues (**Figure 1A**). SIRT1 expression in cancer tissues and normal tissues were 60% and 25%, respectively. Phospho-SIRT1 expression in cancer tissues and normal tissues were 38% and 15%, respectively. The average score of SIRT1 in cancer tissues and normal tissues were 4.780 ± 0.353 (Mean ± SE) and 2.450 ± 0.373, respectively. The average score of Phospho-SIRT1 in cancer tissues and normal tissues were 3.020 ± 0.228 and 1.900 ± 0.261, respectively (**Figure 1B**). Statistical analysis showed that SIRT1 and Phospho-SIRT1 were higher expressed in cancer tissues than normal tissues ($P < 0.05$ for SIRT1, $P = 0.008$ for Phospho-SIRT1).

WB revealed that SIRT1 and Phospho-SIRT1 were both localized in the nucleus of cancer tissues and normal tissues (**Figure 2A**). SIRT1 expression was scored as 1.351 ± 0.020 in cancer tissues and 0.825 ± 0.015 in normal tissues. Phospho-SIRT1 expression was scored as 0.747 ± 0.022 in cancer tissues and 0.381 ± 0.015 in normal tissues (**Figure 2B**). Statistical analysis showed that, the expressions of SIRT1 and Phospho-SIRT1 were higher in cancer tissues than in normal tissues ($P < 0.05$ for SIRT1, $P < 0.05$ for Phospho-SIRT1).

SIRT1 and Phospho-SIRT1 expression in tumor tissues were both related with clinicopathological characteristics

We used the results of IHC to analysis the relationships of SIRT1 and Phospho-SIRT1 expres-

Table 1. The expression of SIRT1 and Phospho-SIRT1 in colorectal cancer

Characteristics	n	Expression of SIRT1			Expression of P-SIRT1		
		Positive	Negative	*P* value	Positive	Negative	*P* value
Gender				0.470			0.190
Male	32	18	14		10	22	
Female	18	12	6		9	9	
Age (year)				0.047			0.547
< 60	21	16	5		9	12	
≥ 60	29	14	15		10	19	
Grade				0.929			0.802
High-Middle	44	27	17		17	27	
Low	6	3	3		2	4	
TNM stage				0.018			0.405
I-II	30	14	16		10	20	
III-IV	20	16	4		9	11	
LN metastasis				0.341			0.285
Absent	31	17	14		10	21	
Present	19	13	6		9	10	
Distant metastasis				NS			NS
Absent	49	30	20		19	31	
Present	1	0	0		0	0	
P53 mutation				0.015			0.186
Positive	27	12	15		8	19	
Negative	23	18	5		11	12	
Ki67				0.108			0.011
Positive	34	23	11		17	17	
Negative	16	7	9		2	14	

P-SIRT1, Phospho-SIRT1; TNM, tumor node metastasis; LN, lymph node. The χ^2 test or Fisher's exact test was used to evaluate the association between SIRT1 or Phospho-SIRT1 expression and clinicopathological characteristics. *P* value < 0.05 was considered statistically significant.

sion with clinicopathological characteristics (**Table 1**). Our experiment results showed that SIRT1 expression correlated with younger age (P = 0.047), advanced TNM stage (P = 0.018) and mutant P53 loss (P = 0.015). Phospho-SIRT1 expression correlated with Ki67 (P = 0.011). Our research demonstrated that SIRT1 may be as both favorable and unfavorable factors and Phospho-SIRT1 may play an important role in colorectal cancer formation.

Phosphorylation of SIRT1 was higher in colorectal cancer tissues than in normal tissues

To investigate the role of Phosphorylation in colorectal cancer, we examined the correlations of Phospho-SIRT1 and SIRT1 in cancer tissues and normal tissues (**Figure 3**). Our experiment results showed that Phospho-SIRT1 and SIRT1 were positively correlated in cancer tissues and normal tissues, and the ratios of Phospho-SIRT1 and SIRT1 were higher in cancer tissues than normal tissues, suggesting that phosphorylation would promote the colorectal cancer formation.

SIRT1 mRNA was no significant difference in colorectal cancer tissues and normal tissues

To determine whether SIRT1 expression was at transcriptional level or at post-transcriptional level, we examined SIRT1 mRNA level in colorectal cancer tissues and normal tissues using Real-Time PCR (**Table 2**). Results showed that the fold change in expression of the SIRT1 gene in cancer tissues compared to the normal tissues is 1.1, suggesting that SIRT1 mRNA has no significant difference in the cancer tissues and normal tissues. We inference that post-transcription may affect the expression of SIRT1 in colorectal cancer tissues.

Discussion

In this study, we examined the expression and subcellular localization of SIRT1 and Phospho-SIRT1 in colorectal cancer tissues and corresponding normal tissues. SIRT1 mRNA level was also examined in cancer tissues and normal tissues using Real-Time PCR. The main results are that: 1. SIRT1 and Phospho-SIRT1 were both localized in the nucleus and overexpressed in cancer tissues. 2. SIRT1 overexpression was associated with younger age, advanced TNM stage and mutant P53 loss, Phospho-SIRT1 was associated with Ki67. 3. Phosphorylation of SIRT1 was higher in colorectal cancer tissues than normal tissues. 4. SIRT1 mRNA level was no significant difference in cancer tissues and normal tissues.

There are many controversies on SIRT1: SIRT1 may be as a tumor promoter or tumor suppressor, SIRT1 may localize in the nucleus or cyto-

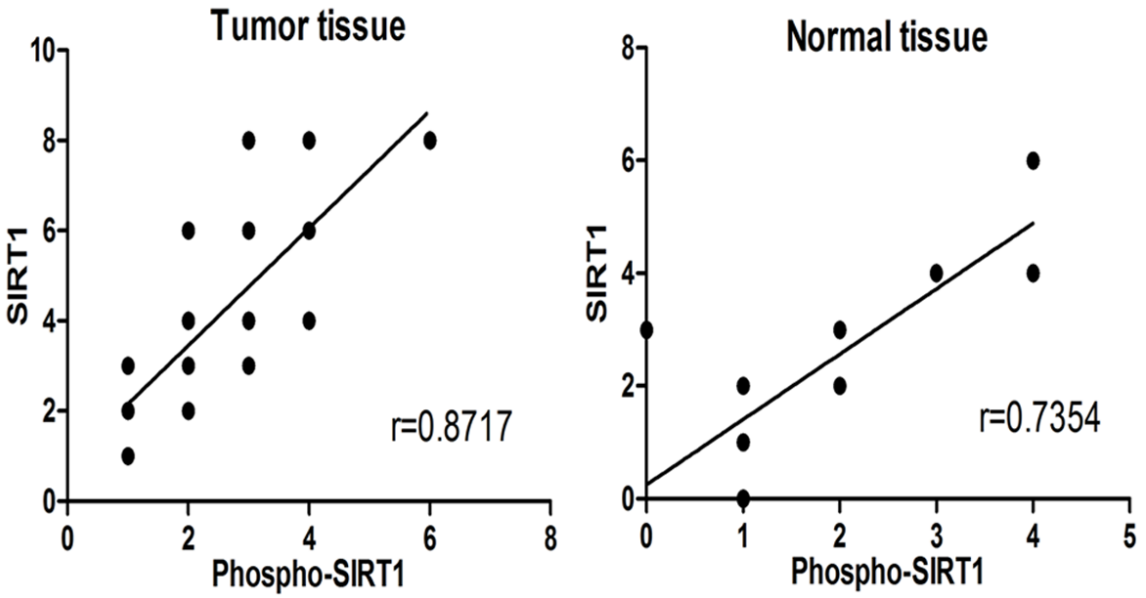

Figure 3. Spearman correlation analysis was used to examine the expression correlation between Phospho-SIRT1 and SIRT1. Phospho-SIRT1 and SIRT1 had a highly positive correlation in both tumor tissues (r = 0.872) and normal tissues (r = 0.735). The ratios of Phospho-SIRT1 and SIRT1 expression in tumor tissues were higher than normal tissues.

Table 2. Relative expression of SIRT1 mRNA in tumor tissues and normal tissue

Tissue	Avg. SIRT1 C_T	Avg. GAPDH C_T	ΔC_T	$\Delta\Delta C_T$	Normalized SIRT1 amount relative to normal $2^{-\Delta\Delta Ct}$
Tumor	27.187 ± 0.165	25.265 ± 0.287	1.922 ± 0.259	0.178 ± 0.259	1.1 (0.7-1.1)
Normal	26.725 ± 0.119	24.981 ± 0.321	1.744 ± 0.299	0.000 ± 0.299	1.0 (0.8-1.2)

The comparative C_T method was used to analyze the expression of SIRT1 mRNA. Avg., Average; ΔC_T = Avg; SIRT1 C_T - Avg; GAPDH C_T; $\Delta\Delta C_T$ = Avg; $\Delta C_{T,Tumor}$ - Avg. $\Delta C_{T,Normal}$. Fold change = $2^{-\Delta\Delta Ct}$.

plasm. Even in the same tumors, such as colorectal cancer, SIRT1 results were various. Walter Stünkel's found that SIRT1 was highly expressed in cancer tissues, and mainly localized in the cytoplasm [7]. Si-Hyong Jang demonstrated that SIRT1 was low expression in colon cancer and located in the nucleus by IHC [24]. Qihuang Jin revealed that SIRT1 in LoVo cell lines might partially localized in the cytoplasm and proved that cytoplasm localization of SIRT1 was transferred from the nucleus [38]. In our study, we observed overexpression and nuclear localization of SIRT1 in colorectal cancer.

Studies have shown that SIRT1 protein level was associated with the mitotic activity regardless of their mRNA level [39]. It also has been demonstrated that phosphorylation might affect the activity and subcellular localization of SIRT1. We hypothesis that whether the phosphorylation status of SIRT1 more directly determine the role of SIRT1 in tumors? Whether the subcellular localization of Phospho-SIRT1 reflects the nucleocytoplasmic shuttling of SIRT1? However, until now, researches on Phospho-SIRT1 in tumors are rare. It is known that SIRT1 have 13 phosphorylation sites and Ser47 is a common one, so we applied Phospho-SIRT1 (pS47) antibody to analysis in detail the expression and subcellular localization of Phospho-SIRT1 in colorectal cancer.

First, to determine whether SIRT1 in colorectal cancer was overexpressed at the transcriptional level or the post-transcriptional level, we evaluated SIRT1 mRNA level using Real-Time PCR. Our data revealed that SIRT1 mRNA level in colorectal cancer tissues and normal tissues was no significant difference, suggesting that post-transcriptional level plays an important role on the regulation of SIRT1 expression.

Results of IHC and WB showed that Phospho-SIRT1 was highly expressed in colorectal cancer and located in the nucleus. After analysis of its relationship with clinicopathological characteristics, we found Phospho-SIRT1 was positively related with Ki67 and had no significant correlation with lymph node metastasis, TNM stage. As we all know, Ki67 was closely related with mitosis and reflects the proliferation of cells, so we believe that Phospho-SIRT1 may promote the tumorigenesis in colorectal cancer.

To confirm the role of Phospho-SIRT1 in cancer, we analyzed the correlation between Phospho-SIRT1 and SIRT1 in cancer tissues and normal tissues. We found that Phospho-SIRT1 and SIRT1 was positively correlated in both cancer tissues and normal tissues, the ratios of Phospho-SIRT1 and SIRT1 were higher in cancer tissues. The results demonstrated that phosphorylation might promote the formation of colorectal cancer.

Phosphorylation has been demonstrated to promote the transfer of SIRT1 to the nucleus. We speculate that SIRT1 may have a dual position of nucleus and cytoplasm, Phospho-SIRT1 may represent the shuttling portion of SIRT1 from the cytoplasm to the nucleus. To our surprise, our results showed that SIRT1 and Phospho-SIRT1 were both located in the nucleus and not in cytoplasmic. We couldn't explain the phenomenon. Maybe SIRT1 localization has a complex regulation. Therefore, we cannot come to the view that phospho-SIRT1 may affect the subcellular localization of SIRT1.

Our experiments have some limitations, such as small sample size, Phospho-SIRT1 antibody selected only for certain sites which is not enough to represent all phosphorylation status of SIRT1, and so on. We cannot exclude the tumor heterogeneity and staining scoring method to interfere the experiment. In short, we need further study on the roles of Phospho-SIRT1 in cancers.

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Disclosure of conflict of interest

None.

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