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Prognostic Significance of Sirtuins Expression in Papillary Thyroid Carcinoma

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Background and Objectives: Sirtuins (SIRTs) play important roles in cellular and organismal homeostasis. They have distinct gene expression patterns in various cancers; however, the relationship between SIRT expression and the progression of thyroid cancer is unclear. We investigated the expression of SIRTs in patients with papillary thyroid carcinoma (PTC) and their role as biomarkers for predicting the aggressiveness of this disease. **Materials and Methods:** We used immunohistochemical staining to evaluate the expression of SIRT1 and SIRT3 in tumor specimens from 270 patients with PTC. We also evaluated the potential association between SIRT expression and diverse clinicopathological features. **Results:** High SIRT1 expression was negatively correlated with lymphovascular invasion, central lymph node metastasis, and lateral lymph node metastasis. Multivariate analyses revealed that high SIRT1 expression was a negative independent risk factor for lateral lymph node metastasis. By contrast, high SIRT3 expression was positively correlated with locoregional recurrence. Interestingly, when patients were grouped by tumor SIRT expression patterns, the group with low SIRT1 expression and high SIRT3 expression was correlated with more aggressive cancer phenotypes including central lymph node metastasis and lateral lymph node metastasis. **Conclusion:** Our results suggest that SIRTs play dual roles in tumor progression, and the combination of decreased SIRT1 expression and increased SIRT3 expression is significantly associated with a poor prognosis in patients with PTC.

Key Words: Papillary thyroid carcinoma, SIRT1, SIRT3, Sirtuin, Tumor progression

Introduction

Sirtuins (SIRTs) are nicotinamide adenine dinucleotide (NAD+)-dependent enzymes associated with ageing-related diseases including neurodegeneration, cardiovascular disease, and various types of cancer.[1)] Seven SIRT proteins (SIRT1-SIRT7) have been identified, which share a core NAD+ binding domain but different enzymatic activities, functions, and subcellular localizations.[2)] Cancer cells exhibit altered metabolism, such as glycolysis and glutaminolysis, to regulate cell growth and cell division.[3)] Previous studies have reported that SIRTs inhibit these processes, countering cancer-associated altered metabolic pathways and uncontrolled cell proliferation.[3)] However, SIRTs also regulate DNA repair, cell cycle, cell survival, and apoptosis, and play significant roles in cancer initiation and progression.[4)] In addition, they play important roles in cancer progression and metastasis by regulating the epithelial-to-mesenchymal transition and cell-to-cell communication.[5)]

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Thyroid cancer cells exhibit increased aerobic glycolysis and suppressed mitochondrial oxidative phosphorylation (OxPhos) for a more advantageous metabolism for cancer cell survival. Normal human thyroid tissues and tumor tissues are capable of a metabolic switch between aerobic glycolysis and OxPhos depending on the microenvironment.[6] Inducing the expression of $BRAF^{V600E}$ suppresses the apoptotic response, increases the rate of glucose uptake, and decreases O2 consumption, which suggests that $BRAF^{V600E}$ reduces mitochondrial OxPhos, a signature feature of cancer cells.[7] These findings suggest that altered metabolic pathways in thyroid cancer may be an important aspect of regulating tumor cell proliferation and tumor progression. However, there have been few studies on the role of SIRTs in thyroid cancer.

Previously, transgenic SIRT1 expression was reported as an oncogene in a Pten-deficient rodent model of thyroid and prostate cancer.[8] Researchers have also observed that SIRT1 expression is positively correlated with c-Myc levels in the human thyroid, and SIRT1 overexpression stabilized c-Myc protein in cultured thyroid cancer cells. Moreover, another research group discovered that the induction of SIRT1 and SIRT3 may determine thyroid cancer cell survival under etoposide-induced genotoxic apoptosis in thyroid cancers,[9] however, there have been no studies on the role of SIRTs as predictive biomarkers in thyroid cancer progression. Here, we report SIRT expression patterns in human papillary thyroid cancer as a biomarker to predict thyroid cancer progression.

Materials and Methods

Patients and Tissue Samples

From 2003 to 2010, 270 patients who underwent total thyroidectomy and cervical lymph node (LN) dissection for the treatment of papillary thyroid cancer (PTC) at the Department of Otolaryngology-Head and Neck Surgery of Chungnam National University Hospital, South Korea, were analyzed retrospectively. Patients were diagnosed with PTC preoperatively using fine needle aspiration cytology, or intraoperatively using frozen tissue sections. All patients underwent central LN dissection. Simultaneous central and lateral LN dissection were performed in 48 patients due to preoperative evidence of metastatic LNs in the lateral neck. Prophylactic central LN dissection was performed in 191 patients without clinical evidence of positive LNs on imaging or palpation, and therapeutic central LN dissection was performed in 31 patients with clinically evident positive central LNs. Lateral LN dissection was performed using a modified radical operation that involved complete removal of levels II-V lateral cervical LNs. Level I dissection was not performed if there was no clinical evidence of metastases at level I. Patients who underwent lobectomy only, but not central LN dissection, or whose medical records were unclear, were excluded from the study. All specimens were collected from patients after informed consent had been obtained in accordance with the institutional guidelines of our hospital. The tumor stage was determined according to the histologic classification of thyroid tumors suggested by the World Health Organization. Surveillance for recurrent disease usually involved a physical examination, measurement of serum levels of thyroglobulin, and ultrasonographic examination every 12 months. We followed the patients for a mean duration of 106.6±22.5 months to evaluate tumor recurrence.

Immunohistochemistry

Thyroid tissue specimens resected from patients were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), washed with PBS, dehydrated with ethanol and xylene, and embedded in paraffin wax. The tissues were retrieved from the archives of the Department of Pathology of Chungnam National University Hospital. Tissues were sectioned at 4 μm and immunohistochemistry (IHC) was performed using the Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, CA, USA) according to the manufacturer's instructions. Antigen retrieval was performed using microwaving tissues in citrate buffer for 10 min. Endogenous peroxidase activity was inactivated by incubation in 3% hydrogen peroxide for 10 min.

Nonspecific binding sites were blocked by incubating in 10% normal goat serum diluted with PBS. Then IHC was performed using rabbit polyclonal anti-SIRT1 antibody (1:50 in blocking solution, 1 h at room temperature; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit polyclonal anti-SIRT3 antibody (1:40 in blocking solution, 1 h at room temperature; Santa Cruz Biotechnology). The staining was assessed by two investigators blinded to the corresponding clinicopathological data. In tumors with multicentricity (n=112), the most dominant tumor nodule was investigated for IHC analyses, and then the entire section was assessed. To quantify SIRT IHC staining, a scoring system was used that combined the intensity and distribution of positive staining: 0, no staining; +1, weak staining in focal tumor areas; +2, moderate staining in most tumors; and +3, strong staining in most tumors (Fig. 1). Finally, for statistical comparisons, tissue slides with scores of 0 or +1 were included in the low SIRT immunoexpression group, and those with scores of +2 or +3 were included in the high SIRT immunoexpression group.

Statistical Analyses

The results are expressed as means±standard deviations (SDs). Fisher's exact test and two-tailed t-tests were used to compare patient clinicopathological data. Patients were divided into two groups, high and low immunostaining, according to SIRT expression scores as described above. Group comparisons of categorical variables were performed using linear-by-linear association and multivariate analyses using stepwise logistic regression. All *in vitro* experiments were repeated three times, and statistical significance was analyzed using two-tailed Student's t-tests or one-way analysis of variance followed by Tukey's post hoc test. Data are presented as the means±SDs, and p values less than 0.05 were considered statistically significant (*$p<0.05$; **$p<0.01$). SPSS software v. 22 (IBM Corp., Armonk, NY, USA) was used for all statistical analyses.

Results

Patient and Tumor Characteristics

The baseline characteristics of the patients are shown in Table 1. The mean age of the study population was 48.3 years (range, 22-84 years). The majority of the patients were female (82.6%). The average size of the primary tumor was 1.1±0.8 cm. The proportion of patients with multicentricity was 42%

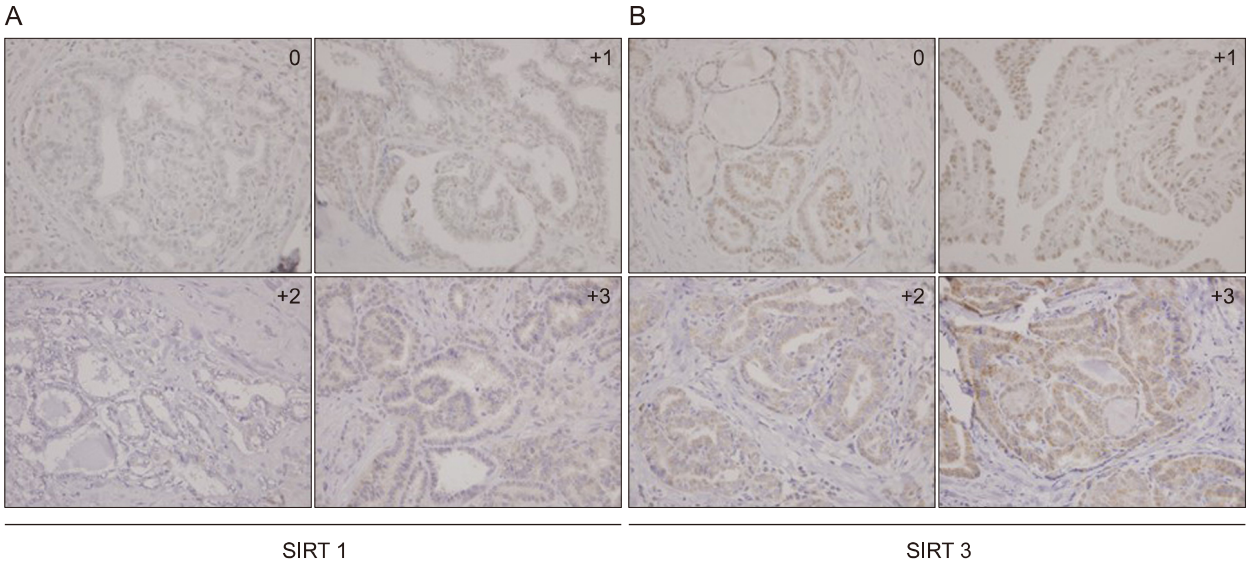

Fig. 1. Immunohistochemical evaluation of sirtuin 1 (SIRT1) and sirtuin 3 (SIRT3) expression in papillary thyroid carcinoma (PTC) tissue. (A) Representative immunohistochemical images of SIRT1. (B) Representative immunohistochemical images of SIRT3. 0: no staining intensity, +1: weak staining intensity, +2: moderate staining intensity, +3: strong staining intensity (magnification ×100)

Table 1. Clinicopathologic parameters of patients (n=270)

Variables		Mean±SD or number of patients (%)
Age, years		48.3±12.4
Gender	Male	47 (17.4)
	Female	223 (82.6)
Tumor size	≤1 cm	110 (40.7)
	>1 cm	160 (59.3)
Multicentricity	No	158 (58.5)
	Yes	112 (41.5)
Microscopic capsular invasion	No	71 (26.3)
	Yes	199 (73.7)
Extrathryoid extension	No	88 (32.6)
	Yes	182 (67.4)
Lymphovascular invasion	No	63 (23.3)
	Yes	207 (76.7)
Lymph node metastasis	No	116 (43.0)
	Yes	154 (57.0)
Central lymph node metastasis	No	116 (43.0)
	Yes	154 (57.0)
Lateral lymph node metastasis	No	222 (82.2)
	Yes	48 (17.8)
Locoregional recurrence	No	233 (86.3)
	Yes	37 (13.7)
Follow-up period (months)		106.6±22.5

SD: standard deviation

(112/270), capsular invasion 73.7% (198/270), extrathyroid extension 67.4% (182/270), and lymphovascular invasion 76.7% (207/270). Of the 270 patients with PTC, the proportions of LN metastases were 57.0% (154/270) and 17.8% (48/270) in the central and lateral compartments, respectively.

Clinicopathological Correlation of SIRT1 Expression in PTC

We analyzed the relationship between clinicopathological parameters and SIRT1 expression in PTC. The patients were divided into two groups according to the SIRT1 expression results. In univariate analyses, high SIRT1 expression was negatively correlated with several clinicopathological aggressive parameters including lymphovascular invasion (p=0.039), and LN metastasis, including both central lymph nodes (p=0.002) and lateral lymph nodes (p<0.001) (Table 2). To identify the role of SIRT1 as an independent predictor of the aggressive phenotypes of PTC, multivariate analyses using stepwise logistic regression was con-

Table 2. Relationships between intensity of sirtuin 1 (SIRT1) staining and clinicopathological factors in 270 patients

Variables		No. of patients	SIRT1		
			Low (Grade 1 and 2)	High (Grade 3 and 4)	p value
Age, years	<45	104	66	38	0.411
	≥45	166	97	69	
Gender	Male	47	31	16	0.389
	Female	223	132	91	
Tumor size	≤1 cm	110	61	49	0.171
	>1 cm	160	102	58	
Multicentricity	No	158	96	62	0.877
	Yes	112	67	45	
Microscopic capsular invasion	No	71	37	34	0.098
	Yes	199	126	73	
Extrathyroid extension	No	88	48	40	0.174
	Yes	182	115	67	
Lymphovascular invasion	No	63	31	32	0.039*
	Yes	207	132	75	
Lymph node metastasis	No	116	58	58	0.002*
	Yes	154	105	49	
Central lymph node metastasis	No	116	58	58	0.002*
	Yes	154	105	49	
Lateral lymph node metastasis	No	222	122	100	<0.001*
	Yes	48	41	7	
Locoregional recurrence	No	233	139	94	0.547
	Yes	37	24	13	

*p<0.05 between the two categories for a given variable

Table 3. Multivariate analysis of the relationship between SIRT1 staining and clinicopathologic factors

Factors	Exp (β)	SE	95.0% CI	p value
Lymphovascular invasion	0.645	0.309	(0.352, 1.182)	0.156
Lateral lymph node metastasis	0.233	0.443	(0.097, 0.555)	0.001*
Central lymph node metastasis	0.681	0.275	(0.345, 0.849)	0.162

Data analyzed using a stepwise logistic
CI: confidence interval, Exp (β): odds ratio, SE: standard error
*p value<0.05

Table 4. Relationships between intensity of sirtuin 3 (SIRT3) staining and clinicopathological factors in 270 patients

Variables		No. of patients	SIRT3		p value
			Low (Grade 1 and 2)	High (Grade 3 and 4)	
Age, years	<45	104	52	52	0.175
	≥45	166	69	97	
Gender	Male	47	27	20	0.055
	Female	223	94	129	
Tumor size	≤1 cm	110	54	56	0.241
	>1 cm	160	67	93	
Multicentricity	No	158	66	92	0.232
	Yes	112	55	57	
Microscopic capsular invasion	No	71	34	37	0.544
	Yes	199	87	112	
Extrathryoid extension	No	88	43	45	0.352
	Yes	182	78	104	
Lymphovascular invasion	No	63	26	37	0.518
	Yes	207	95	112	
Lymph node metastasis	No	116	48	68	0.325
	Yes	154	73	81	
Central lymph node metastasis	No	116	48	68	0.325
	Yes	154	73	81	
Lateral lymph node metastasis	No	222	95	127	0.151
	Yes	48	26	22	
Locoregional recurrence	No	233	111	122	0.019*
	Yes	37	10	27	

* means p value <0.05

ducted on parameters that were shown to be significant in univariate analyses. In multivariate analyses, high SIRT1 expression had independent negative correlation with lateral lymph node metastasis (p=0.001, OR=0.233) (Table 3).

Clinicopathological Correlations of SIRT3 Expression in PTC

Next, we analyzed the relationships between clinicopathological parameters and SIRT3 expression in PTCs. Patients were divided into two groups according to SIRT3 immunoreactivity. In univariate analyses, high SIRT3 expression was significantly associated with locoregional recurrence (p=0.019) (Table 4). Thus, SIRT3 positivity was highly associated with markers of tumor aggressiveness.

Association of Low SIRT1 and SIRT3 Expression with Poor Prognosis in PTC

To evaluate the significance of heterogeneous expression patterns of SIRTs, we analyzed the clinicopathological findings in relation to both SIRT1 and

Table 5. Relationships between patterns of sirtuin staining intensity and clinicopathological factors

Variables		SIRT1 low SIRT3 low (n=88)	SIRT1 low SIRT3 high (n=75)	SIRT1 high SIRT3 low (n=33)	SIRT1 high SIRT3 high (n=74)	p value
Age, years	<45	40	26	12	26	0.444
	≥45	48	49	21	48	
Gender	Male	12	14	11	10	0.368
	Female	76	43	40	64	
Tumor size	≤1 cm	35	26	19	30	0.168
	>1 cm	53	49	14	44	
Multicentricity	No	49	47	17	45	0.648
	Yes	39	28	16	29	
Microscopic capsular invasion	No	23	14	11	23	0.265
	Yes	65	61	22	51	
Extrathryoid extension	No	28	20	15	25	0.289
	Yes	60	55	18	49	
Lymphovascular invasion	No	17	14	9	23	0.214
	Yes	71	61	24	51	
Lymph node metastasis	No	30	28	18	40	0.025*
	Yes	58	47	15	34	
Central lymph node metastasis	No	30	28	18	40	0.025*
	Yes	58	47	15	34	
Lateral lymph node metastasis	No	64	58	31	69	0.001*
	Yes	24	17	2	5	
Locoregional recurrence	No	79	60	32	62	0.072
	Yes	9	15	1	12	

* means p value <0.05

SIRT3 expression. Because our results revealed that low SIRT1 and high SIRT3 expression was correlated with aggressive tumor phenotypes, we compared clinicopathological parameters among 4 groups in relation to both SIRT1 and SIRT3 expression. The group with low SIRT1 and high SIRT3 expression exhibited more aggressive phenotypes such as central lymph node metastasis and lateral lymph node metastasis (Table 5).

Discussion

We identified the different roles of SIRT1 and SIRT3 in predicting tumor progression in papillary thyroid carcinoma. High SIRT1 expression was negatively correlated with aggressive phenotypes, and high SIRT3 expression was positively associated with locoregional recurrence in patients with PTC.

Seven mammalian sirtuins (SIRT1–SIRT7) have been identified with diverse subcellular localizations.[10] Previous studies have suggested that SIRT1 protects against diverse metabolic damage, and prevents ageing-related pathological conditions.[11,12] However, in tumorigenesis, the role of SIRT1 is poorly understood, as data exist to support its role as both a tumor suppressor and tumor promoter.[13] These opposing roles of SIRT1 are related to the significant regulatory roles of diverse genes, including tumor promoters and tumor suppressors, through deacetylating non-histone proteins and methylating DNA.[13] Previous studies in cancer tissues and cancer cell lines have demonstrated that increased SIRT1 protein expression promotes tumor cell proliferation and SIRT1 silencing induced growth arrest and apoptosis of human epithelial cells.[14–17] By contrast, there is much evidence supporting the role of SIRT1 as a tumor suppressor.[18,19] SIRT1 expression was reduced in breast cancer 1 mutant cancer cells, and SIRT1 inhibited survivin, an important anti-apoptotic protein.[20,21] These contradictory roles of SIRT1 in tumorigenesis suggest that it may play a dual role depending on its spatial and temporal distribution in different tissues.

SIRT3, one of the mitochondrial SIRTs, is a key mitochondrial deacetylase that is critical for the preservation of mitochondrial integrity and function.[22] SIRT3 knockout mice exhibited hyperacetylated mitochondrial proteins and reduced adenosine triphosphate (ATP) levels.[23] In addition, under genotoxic stress, mitochondrial SIRT3 was necessary to protect against cell death.[24] SIRT3 also functions as a tumor promoter in multiple cancer pathways.[25,26] However, SIRT3 was also shown to induce growth arrest and apoptosis in several colorectal carcinoma and osteosarcoma cell lines, and in lung fibroblast cells.[27] These results correlate with the role of SIRT3 as a modulator of the JNK2 signaling pathway, and researchers have observed that JNK2 and SIRT1 function as constitutive suppressors of apoptosis in colorectal carcinoma.[28] Therefore, SIRT1 and SIRT3 have opposite roles in colorectal cancer, and these observations are similar to our results.

Our results showed that SIRT1 has a tumor suppressor role and that SIRT3 has the opposite role as a tumor promoter. We suggest that these opposing results may be induced by altered cancer metabolism in thyroid cancer tissues. SIRT1 and SIRT3 expression was detected in most thyroid tumor tissues in our study. However, tumor tissues with aggressive phenotypes showed different expression patterns of SIRT1 and SIRT3 compared to tumor tissues with indolent phenotypes. Tumors with aggressive phenotypes showed lower SIRT1 and higher SIRT3 expression compared to tumors with indolent behavior. Previously, we observed reduced OxPhos and increased ROS levels in thyroid cell lines with $BRAF^{V600E}$ induction.[7] Interestingly, the $BRAF^{V600E}$ mutation was localized in the mitochondria, and altered metabolism was not rescued by ERK or MET inhibitors.[7] In addition, we observed a reduced oxygen consumption rate in thyroid tumor cells, compared to that in normal thyroid cells (unpublished data). These findings suggest that altered metabolic pathways may be related with thyroid tumor aggressiveness. SIRT3 is the major mitochondrial deacetylase driving fatty acid oxidation, suppressing ROS production, and protecting against obesity-induced metabolic deregulation, oxidative stress, and cancer.[4,29,30] We hypothesized that thyroid cancer cells with altered metabolic pathways and increased ROS levels may induce the expression of SIRT3 to preserve tumor cell survival by attenuating ROS, although we were unable to evaluate ROS levels or mitochondrial function in the SIRT3 high expression group. Our data also suggest the role of SIRT1 as a tumor suppressor. Many recent human studies have investigated the role of SIRT1 expression, but the role of SIRT1 in various tumors is controversial. Reduced SIRT1 expression has been reported in human breast cancer, glioblastoma, bladder cancer, ovarian cancer, and oral squamous cell carcinoma.[19] However, two studies on liver cancer showed opposite roles, and multiple studies have identified SIRT1 as an oncogene in diverse cancers.[31,32] Previous reports using transgenic Sirt1 expression in a murine model of thyroid cancer by Pten deficiency demonstrated that SIRT1 expression increases c-Myc transcription, and SIRT1 is overexpressed in thyroid tumor tissues and positively correlates with c-Myc protein levels. Researchers have compared the protein expression of SIRT1 among 29 samples from patients with PTC and 18 samples from control participants.[8] Another study investigated SIRT1 induction by etoposide-induced genotoxic apoptosis in thyroid tumors, and observed the heterogeneous expression of SIRT1 between tumor tissues and normal tissues.[9] We also observed the expression patterns in normal thyroid lesions and thyroid tumor tissues; however, SIRT1 expression was very heterogeneous, and we did not find significant changes between normal tissues and tumor tissues. Interestingly, we discovered that decreased SIRT1 expression was significantly correlated with tumor aggressiveness, including LN metastasis, between groups with high SIRT1 expression and low SIRT1 expression. These results suggest that SIRT1 may play a tumor suppressive role in human thyroid tumorigenesis.

There were several limitations to our study. We were unable to evaluate the exact mechanism of the diverse roles of SIRT1 and SIRT3 in thyroid cancer, or to identify changes in cancer metabolic pathways in our samples. To overcome these issues, *in vivo*

studies using a physiological thyroid cancer model and overexpression or knockout of SIRT1 and SIRT3 are necessary.

In conclusion, we compared clinical parameters in relation to SIRT1 and SIRT3 expression levels in thyroid cancer tissues for the first time, and discovered that SIRTs may serve as predictive biomarkers in thyroid cancer.

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