ORIGINAL ARTICLE

Prognostic Role of SIRT1 in Hepatocellular Carcinoma

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Abstract

Objective: To determine the clinical significance of silent mating type information regulation 2 homolog 1 (SIRT1) expression in Hepatocellular Carcinoma (HCC) and its association with P53 and Yes-associated protein 2 (YAP2) expression.
Study Design: Observational study.
Place and Duration of Study: Department of General Surgery, China-Japan Union Hospital of Jilin University, Changchun, China, from January 2000 to January 2010.
Methodology: Tissue microarray technique and immunohistochemistry were conducted to detect the expression of SIRT1, P53 and YAP2 proteins in 300 self-paired HCC samples. Associations with clinicopathologic manifestations were analyzed, overall survival analysis and multivariate analysis were performed.
Results: By tissue microarray technique and immunohistochemistry on 300 self-paired HCC samples, it was found that SIRT1, P53 and YAP2 were significantly overexpressed in HCC tumor tissues compared with adjacent non-tumor tissues. SIRT1 immunostaining localized both in the nucleus (145/300, 48.3%) and the cytoplasm (70/300, 23.3%), and the over-expression of nuclear SIRT1 was positively related to the overexpression of P53 and YAP2. Survival analysis showed that nuclear SIRT1, P53 and YAP2 overexpression predicted poor overall survival while cytoplasmic SIRT1 overexpression predicted longer overall survival. Multivariate analysis showed nuclear SIRT1 and P53 overexpression as independent tumor promoters while cytoplasmic SIRT1 overexpression as an independent tumor suppressor.
Conclusion: SIRT1 was overexpressed in HCC and the expression was positively related to P53 and YAP2 expression. As the nuclear SIRT1 functions as a tumor promoter and cytoplasmic SIRT1 functions as a tumor suppressor, the role of SIRT1 in HCC should be reconsidered.

Key Words: *Hepatocellular carcinoma. Immunohistochemistry. P53. SIRT1. YAP2.*

INTRODUCTION

Hepatocellular Carcinoma (HCC) is the fifth most common cancer and ranks the third among the most lethal cancers worldwide.[1] The molecular changes and mechanisms that regulate the development and progression of HCC remain largely unclear.[1]

Silent mating type information regulation 2 homologue 1 (SIRT1), a NAD^+-dependent class III deacetylase, is a key modulator in the process of stress response, cell metabolism, aging, apoptosis and cell proliferation.[2] Recently, it is suggested that SIRT1 plays a controversial role in tumorigenesis with functions in both tumor promoting and tumor suppressing.[3] For its deacetylation of the tumor suppressor p53 leading to p53 inactivation and inhibition of apoptosis, SIRT1 initially was suggested as a tumor promoter.[4] Overexpression of SIRT1 was reported in various malignancies, including prostate cancer,[5] ovarian cancer,[5] breast carcinomas,[6] non-melanoma skin cancers,[7,8] acute myeloid leukemia,[9] gastric cancer[10] and colon cancer.[3] On the other hand, reduction of SIRT1 expression was reported in bladder cancer[11] and glioblastoma.[3] The role of SIRT1 in HCC also remains controversial. Overexpression of SIRT1 was reported by several publications,[2,12] however, Wang *et al*.[7] demonstrated that SIRT1 expression in HCC is significantly reduced by the analysis of microarray data. Furthermore, the cytoplasmic SIRT1 expression was mentioned by several publications,[6,13] however, the clinical significance of the cytoplasmic SIRT1 has never been described in the previously published literature.

The purpose of this study was to determine the clinical significance of SIRT1 expression in HCC and its association with P53 and YAP2 expression.

METHODOLOGY

A retrospective HCC sample series which underwent initial surgical resection between January 2000 and January 2010, was collected from the China-Japan Union Hospital of Jilin University (Changchun, China). The study was approved by the local Institutional Review Board of the China-Japan Union Hospital and informed consents from all patients or their relatives were obtained. The exclusion criteria were radiotherapy or chemotherapy administered before surgical resection, liver transplantation, lost to follow-up and death from other reasons. The formalin-fixed and paraffin-embedded

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HCC samples were obtained from the Pathology Department and all the diagnoses of HCC were pathologically reconfirmed.

The clinicopathological information for all cases was retrieved from the Clinical Database of our hospital. Tumor size was defined as the largest tumor diameter which was measured on the surgical tumor specimen; tumor stage was evaluated according to the American Joint Committee on Cancer (AJCC) criteria,[14] tumor differentiation were graded according to the World Health Organization International Histological Classification of Tumors; venous infiltration was defined based on the final histological examination; peplos infiltration was defined as the peplos infiltrated by at least 2/3 in depth. Overall survival time was calculated from surgery to death.

Tissue microarray (TMA) were constructed from the 300 self-paired formalin-fixed and paraffin-embedded HCC samples (4 um sections). The adjacent non-tumor tissues (NT) were collected at least 2 cm from the tumor edge. Immunohistochemistry of SIRT1, P53 and YAP2 were performed by the PV9000 method. After deparaffinization and rehydration, epitope retrieval was performed in citrate buffer (pH 6.0) at 120°C for 2.5 min. Peroxidase blocking procedure performed and slides were incubated with 10% normal goat serum for 30 min, and then incubated with the primary antibody at 4°C overnight (SIRT1 antibody: Santa cruz, diluted 1:75; P53 antibody: Santa cruz, diluted 1:50; YAP antibody: Santa cruz, diluted 1:100). Slides were washed by phosphate-buffered saline and incubated with signal enhancer for 30 min, and then incubated with HRP-conjugated secondary antibody at room temperature for 30 minutes. Slides were washed and incubated within 3,3'-diaminobenzidine substrate and counterstained with hematoxylin. Negative control was performed by the replacement of the primary antibody with 10% normal goat serum.

The intensity of immunohistochemistry was graded by the H-Score system in which the score was calculated by multiplying the intensity of the stain times the area of the stain. The staining intensity was graded as 0 - (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining) and the area percentage of each intensity were evaluated, and then the H score was calculated by the sum of the area percentage of 1+, twice the area percentage 2+, and thrice the area percentage 3+. H score was graded from 0 to 3 and we interpreted > 0 as positive expression.

Analysis were performed using the Statistical Package for Social Sciences (SPSS) version 17 software. Associations between SIRT1, P53, YAP2 expression and clinical characteristics were analyzed by chi-square test, Student's t-test or Mann-Whitney U-test. Overall survival analysis was performed by the Kaplan-Meier method using log-rank test. Multivariate logistic regression analysis was performed using the Cox proportional hazards model, with $p < 0.05$ included and $p > 0.10$ excluded using a back Wald method. Two-sided $p < 0.05$ was considered of statistical significance.

RESULTS

Three hundred cases, as summarized in Table I, there were 267 males and 33 females and the mean age was 53.0 ± 10.6 years (18 - 79 years) were studied. Follow-up period ranged from 40 months to 136 months, with a median of 62.0 months (mean 64.0 months), 164 (54.7%) cases died at the end of follow-up.

The representative immunohistochemical photomicrographs of SIRT1, P53 and YAP2 staining was shown in Figure 1A. Immunostaining of SIRT1 in HCC tumor tissues (T) was mainly located in the nucleus (145/300 cases) while cytoplasmic SIRT1 could be detected in 70/300 cases (nucleus 48.3% vs. cytoplasm 23.3%: $p < 0.001$). Shown in Figure 1B, SIRT1 was only expressed in the nucleus of case 3 while SIRT1 was only expressed in the cytoplasm of case 4. In adjacent NT, the nuclear SIRT1 was detected in 67/300 cases and cytoplasmic SIRT1 77/300 (nucleus 22.3% vs. cytoplasm 25.7%: p=0.339). Shown in Figure 1C, the H-score of nuclear SIRT1 staining in T group was significantly higher than that in NT group (0.633 ± 0.808 vs. 0.155 ± 0.383, $p < 0.001$) while cytoplasmic SIRT1 not. P53 immunostaining was only located in the nuclei of HCC tumor tissue, the positive rate was 109/300 (36.3%), no expression of P53 was found in NT. YAP2 could also be located both in nucleus and cytoplasm. The nuclear YAP2 positive rate in T was 181/300 (60.3%) and in NT was 25/300 (8.3%), $p < 0.001$. The cytoplasmic YAP2 positive rate in T was 134/300 (44.7%) and in NT was 19/300 (6.3%, $p < 0.001$).

In poor cellular differentiated cases, overexpression of SIRT1 together with overexpression of P53 and YAP2 could usually be found, meanwhile, in the moderate and high cellular differentiated cases, low or negative expression of SIRT1, P53 and YAP2 could usually be detected. Statistically SIRT1 expression was significantly related to the expression of P53 and YAP2 (SIRT1 and P53: correlation factor 0.264, $p < 0.001$; SIRT1 and YAP2: correlation factor 0.384, $p < 0.001$). Shown in Figure 1D, overexpression of P53 could be found in nuclear SIRT1 positive group compared with nuclear SIRT1 negative group (0.561 ± 0.802 vs. 0.321 ± 0.538), and the calculated p-value was 0.025 using Mann-Whitney test. Shown in Figure 1E, over expression of YAP2 could be found both in nucleus and cytoplasm in nuclear SIRT1 positive group compared with nuclear SIRT1 negative group (nucleus: 0.600 ± 0.648 vs. 0.180 ± 0.246; cytoplasm: 0.517 ± 0.588 vs. 0.090 ± 0.230), and the calculated p-value were $p < 0.001$ and $p < 0.001$ using Mann-Whitney test.

Table I: Relation of nuclear SIRT1 overexpression with clinicopathological characteristics in HCC.

	Total cases	Nuclear SIRT1 expression		
		Negative	Positive	p-value[a]
		155 (51.7%)	145 (48.3%)	
Age (mean ± SD, years)	53.0±10.6	53.5±10.5	52.4±10.7	0.371
Tumor size (cm)	6.1±3.8	6.2±3.7	6.1±4.0	0.479
Median survival time (95% CI, months)	53.9 (46.4-61.5)	62.1 (43.6-80.5)	45.1 (36.5-53.7)	0.017*
Gender				
Male	267	143 (53.6%)	124 (46.4%)	0.062
Female	33	12 (36.4%)	21 (63.6%)	
Hepatitis B				
Absent	53	30 (56.6%)	23 (43.4%)	0.452
Present	247	125 (50.6%)	122 (49.4%)	
Hepatitis C				
Absent	288	145 (50.3%)	143 (49.7%)	0.036*
Present	12	10 (83.3%)	2 (16.7%)	
Serum AFP (ng/mL)				
< 400	190	107 (56.3%)	83 (43.7%)	0.030*
≥ 400	96	41 (42.7%)	55 (57.3%)	
Tumor nodules				
Single	243	128 (52.7%)	115 (47.3%)	0.471
Multi	57	27 (47.4%)	30 (52.6%)	
Cellular differentiation				
Well	27	17 (63.0%)	10 (37.0%)	0.002*
Moderate	230	126 (54.8%)	104 (45.2%)	
Poor	43	12 (27.9%)	31 (72.1%)	
AJCC tumor stage				
I	11	6 (54.5%)	5 (45.5%)	0.547
II	129	71 (55.0%)	58 (45.0%)	
III	114	59 (51.8%)	55 (48.2%)	
IV	46	19 (41.3%)	27 (58.7%)	
Venous infiltration				
Absent	147	80 (54.4%)	67 (45.6%)	0.349
Present	153	75 (49.0%)	78 (51.0%)	
Pre-operative local metastasis and infiltration				
Absent	202	111 (55.0%)	91 (45.0%)	0.102
Present	98	44 (44.9%)	54 (55.1%)	
Peplos infiltration				
No infiltration	99	58 (58.6%)	41 (41.4%)	0.044*
Infiltration	127	68 (53.5%)	59 (46.5%)	
No peplos formation	73	29 (39.7%)	44 (60.3%)	
Adjacent non-tumor liver status				
Noncirrhotic	6	1 (16.7%)	5 (83.3%)	0.025*
Chronic hepatitis	52	34 (65.4%)	18 (34.6%)	
Cirrhotic	241	119 (49.4%)	122 (50.6%)	
Peripheral fatty degeneration				
Absent	156	84 (53.8%)	72 (46.2%)	0.826
Mild	80	42 (52.5%)	38 (47.5%)	
Moderate	32	15 (46.9%)	17 (53.1%)	
Severe	30	14 (46.7%)	16 (53.3%)	
P53 expression				
Negative	191	108 (56.5%)	83 (43.5%)	0.025*
Positive	109	47 (43.1%)	62 (56.9%)	
YAP2 expression				
Negative	119	71 (59.7%)	48 (40.3%)	0.025*
Positive	181	84 (46.4%)	97 (53.6%)	

*SIRT1 = Silent mating type information regulation 2 homolog 1; AJCC = American Joint Committee on Cancer; SD = Standard deviation; AFP = α-fetoprotein; CI = confidence interval. * Statistically significant. [a] P-value of age was calculated by Student's t test, tumor size by Mann-Whitney U-test, survival time by Log Rank test, and others by chi-square test.*

Table II: Cox regression analysis of overall survival.

Variables	Univariate analysis[a]	Multivariate analysis	
	p-value	Hazards ratio (95% CI)	p-value
AJCC tumor stage (I, II, III, IV)	<0.001*	1.404 (1.146-1.72)	0.001*
Pre-operative local metastasis and infiltration (absent or present)	<0.001*	1.747 (1.148-2.66)	0.009*
Nuclear SIRT1 expression (negative or positive)	0.017*	1.418 (1.024-1.965)	0.035*
Cytoplasmic SIRT1 expression (negative or positive)	0.005*	0.615 (0.408-0.928)	0.020*
P53 expression (negative or positive)	0.005*	1.612 (1.16-2.241)	0.004*
Tumor size (small, moderate or large)	<0.001*	1.224 (0.987-1.518)	0.065
Tumor nodules (single or multi)	<0.001*	1.462 (0.978-2.186)	0.064
Peplos infiltration (absent, present or no peplos formation)	0.003*	-	0.245
Serum AFP level (< 400 or ≥ 400 ng/mL)	0.029*	-	0.262
Cellular differentiation (well, moderate or poor)	<0.001*	-	0.545
Venous infiltration (absent or present)	<0.001*	-	0.839

*SIRT1 = Silent mating type information regulation 2 homolog 1; AJCC = American Joint Committee on Cancer; AFP = α-fetoprotein; CI = Confidence interval. * Statistically significant. [a] Univariate Analysis was performed using Kaplan Meier analysis.*

As presented in Table I, overexpression of SIRT1 was significantly associated with short survival time, hepatitis C infection, high serum AFP, peplos infiltration, low cellular differentiation, adjacent NT liver cirrhosis, P53 expression and YAP2 expression. Although data not shown, P53 overexpression was associated with venous infiltration (p=0.001), pre-operative local metastasis and infiltration (p=0.004), peripheral fatty degeneration (p=0.026) and low cellular differentiation (p=0.006). High nuclear YAP2 expression was associated with high serum AFP (p=0.039), low cellular differentiation (p < 0.001), number of tumor nodules (p=0.026), pre-operative local metastasis and infiltration (p=0.006) and AJCC tumor stage (p < 0.001).

As shown in Figure 2A, nuclear SIRT1 overexpression in T was significantly associated with short overall survival (p=0.017). The median survival time for the positive and negative groups were 45.1 months (95% CI: 36.5 - 53.7) and 62.1 months (95% CI: 43.6 - 80.5). Shown in Figure 2B, cytoplasmic SIRT1 overexpression in T was significantly associated with longer overall survival (p=0.005). No prognostic significance of SIRT1 expression in NT could be detected.

To further explore the significance of SIRT1 expression as well as the nuclear SIRT1 expression in NT, the self-paired T and NT immunoreactivity was combined by a calculated variable of ΔSIRT1 (the elevated SIRT1 expression in T compared with that in NT). As shown in Figure 2C, nuclear ΔSIRT1 was significantly associated with poor overall survival (p=0.009). The oncogenic role of nuclear SIRT1 and the protective role of cytoplasmic

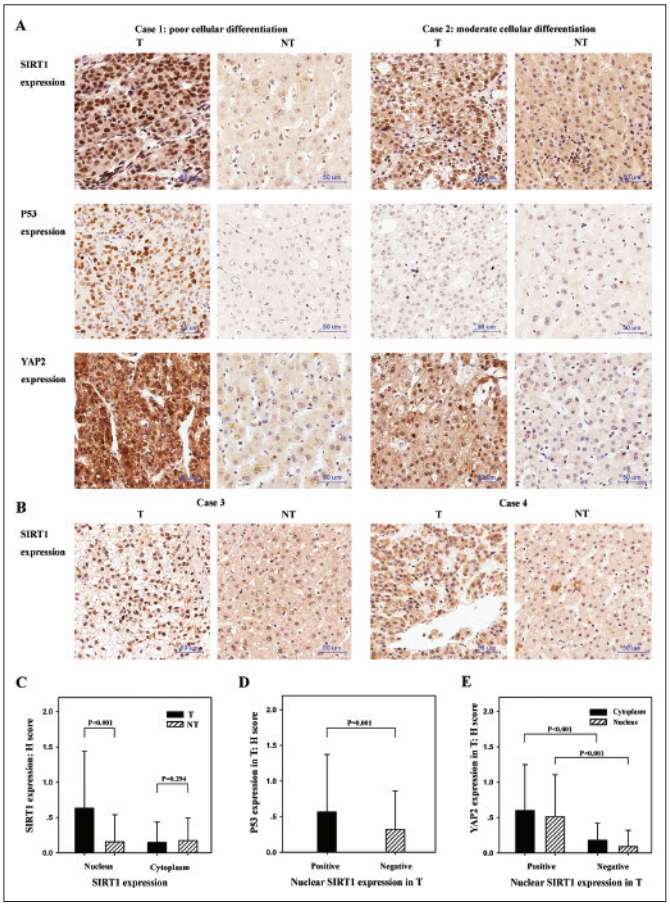

Figure 1 (A,B,C,D,E): Immunohistochemistry: overexpression of SIRT1 and the associated over expression of YAP2 and P53 expression in HCC (T: tumor tissues; NT: adjacent non-tumor tissues; immunoreactivity was graded by H-score; means, standard deviation and p-values were shown, T test). **(A)** Representative immunohistochemical photomicrographs of SIRT1, YAP2 and P53 staining in poor cellular differentiation (case 1) and moderate cellular differentiation (case 2). **(B)** Representative immunohistochemical photomicrographs of SIRT1 staining. Case 3: positive nuclear & negative cytoplasmic SIRT1 expression in T. Case 4: negative nuclear & positive cytoplasmic SIRT1 expression in T. **(C)** Nuclear SIRT1 overexpression in T compared with NT was detected while cytoplasmic SIRT1 not. **(D)** P53 overexpression was detected in nuclear SIRT1 positive group compared with nuclear SIRT1 negative group (Mann-Whitney test). **(E)** YAP2 over-expressions both in nucleus and cytoplasm were detected in nuclear SIRT1 positive group compared with nuclear SIRT1 negative group (Mann-Whitney test).

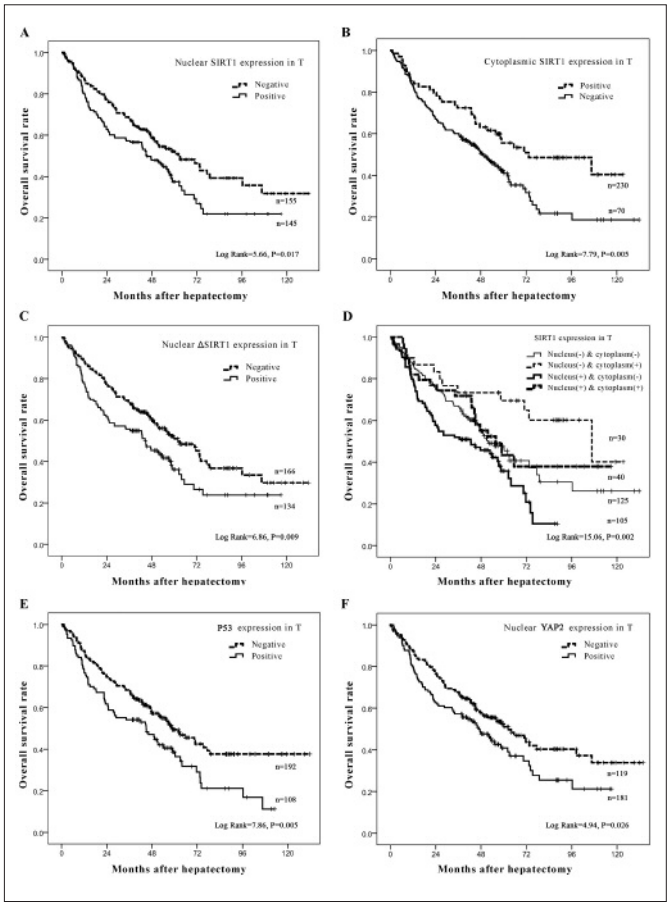

Figure 2 (A,B,C,D,E,F): Kaplan-Meier overall survival analysis: over expressions of nuclear SIRT1, P53 and YAP2 were associated with poor overall survival while cytoplasmic SIRT1 overexpression was associated with longer survival. **(A)** Nuclear SIRT1 overexpression in tumor tissues (T) was associated with poor overall survival. **(B)** Cytoplasmic SIRT1 overexpression in T was associated with longer overall survival. **(C)** Defined as the elevated nuclear SIRT1 expression in T compared with self-paired adjacent non-tumor tissue (NT), nuclear ΔSIRT1 was significantly associated with poor overall survival. **(D)** Divided into 4 groups according to the nuclear and cytoplasmic SIRT1 immunoreactivity, the group of positive nuclear & negative cyto-plasmic SIRT1 expression was associated with poor overall survival. **(E)** P53 overexpression was associated with poor overall survival. **(F)** Nuclear YAP2 overexpression was associated with poor overall survival.

SIRT1 was further analyzed by the division of HCC cases into 4 groups. As shown in Figure 2D, positive nuclear SIRT1 and negative cytoplasmic SIRT1 group (n=105, representative immunohistochemical staining was shown in Figure 1B left) was associated with poor overall survival while negative nuclear and positive cytoplasmic SIRT1 group (n=30). Representative immuno-histochemical staining was shown in Figure 1B right). It was associated with longer survival time (p=0.002).

As shown in Figure 2E and 2F, P53 overexpression and nuclear YAP2 overexpression in T were all significantly associated with poor overall survival (P53: p=0.005; YAP2: p=0.026).

To explore the independent prognostic markers of HCCs, variables of statistical significance in univariate overall survival analysis were included in the Cox regression model. Shown in Table II, of the 11 variables included, 5 were identified as independent prognostic markers, including AJCC tumor stage, pre-operative local metastasis and infiltration, nuclear SIRT1 expression, cytoplasmic SIRT1 expression and P53 expression. Of the above 5 markers, only the cytoplasmic SIRT1 expression was a protective marker.

DISCUSSION

The purpose of the present study was to investigate the clinical significance of SIRT1 expression and its association with P53 and YAP2 expression in HCC. It was found that SIRT1 immunostaining could be located both in the nucleus (145/300, 48.3%) and the cytoplasm (70/300, 23.3%), and the overexpression of nuclear SIRT1 was positively related to the overexpression of P53 and YAP2. Survival analysis and multivariate

analysis showed nuclear SIRT1 overexpression as independent tumor promoters while cytoplasmic SIRT1 overexpression as an independent tumor suppressor.

Despite extensive studies in the last few decades, the role of SIRT1 in tumorigenesis remains controversial. The findings that overexpression of SIRT1 reduces intestinal tumorigenesis and colon cancer formation suggest that SIRT1 may serves as a tumor suppressor.[15] SIRT1 overexpression predicts a favorable prognosis in ovarian cancer[5] and head and neck squamous cell carcinomas,[13] despite being overexpressed. On the other hand, SIRT1 deacetylation mediated inhibition of tumor suppressors such as P53, Rb, Ku70 and FoxO3, suggest that SIRT1 may serves as a tumor promoter.[1,3] It was reported that SIRT1 overexpression was associated with poor prognosis in gastric cancer, breast cancer, malignant lymphoma and diffuse large B cell lymphoma.[9]

The role of SIRT1 in HCC is still controversial. Molecular studies showed that in liver cancer cells SIRT1 inactivation induced in vitro cell growth and proliferation via the induction of apoptosis or cellular senescence.[12,16,17] In clinical studies, SIRT1 nuclear expression rate was reported ranging from 30.7%[16] to 56%[1] (in the present study it is 48.3%). Although the oncogenic role of SIRT1 in HCC is generally supported in clinical studies,[1,4,11,12,16] Wang *et al*.[7] analyzed 263 HCCs on a microarray and found that SIRT1 expression was reduced in 42/263 samples and suggested that SIRT1 might act as a tumor suppressor through its role in DNA damage response and genome integrity.

SIRT1 immunoreactivity was initially reported as predominated localized in nuclei but recently cytoplasmic SIRT1 was also reported.[6,13,18,19] It was suggested that the cytoplasm-localized SIRT1 was mainly from nuclei[20] and enhanced caspases-dependent apoptosis.[20,21] Stunkel *et al*.[18] reported that SIRT1 overexpression was predominantly localized in the cytoplasm in SW620 and HeLa cells as well as colorectal cancers. Ohsawa *et al*. reported that cytoplasmic SIRT1 was expressed in 59/122 breast carcinomas and was associated with poor prognosis.[6] In HCC, although the cytoplasmic SIRT1 was mentioned in several publications,[1,2,16] the expression rate and the prognostic significance was never explored. In the present study. We found that cytoplasmic SIRT1 was expressed in 70/300 (23.3%) HCCs and was associated with a favorable prognosis.

Many transcriptional factors have been identified as the targets of SIRT1, including P53, E2F1, FOXO, NF-κB, Ku70, c-Myc and histones.[2,3,20] P53, which plays an important role in regulating cellular senescence and apoptosis,[11] physically interacts and is deacetylated by SIRT1 and induces the repression of p53 dependent apoptosis in response to DNA damage.[22] P53 loss or mutation could be detected in over half of all the cancers and was associated with tumorigenesis. The correlation of SIRT1 and P53 expression was explored in the present study and we found that P53 was over-expressed in HCCs and was an independent tumor promoter, and the overexpression of P53 was significantly correlated with the overexpression of SIRT1.

Yes-Associated Protein 2 (YAP2), a downstream effector of the Hippo signalling pathway that controls cell growth and organ size,[2] was reported to play an important role in HCC.[23] A recent study revealed YAP2 was associated with SIRT1 in the tumorigenesis of HCC in fundamental experiments.[2] The clinical association of SIRT1 and YAP2 was explored in the present study and we conformed that nuclear YAP2 was overexpressed and significantly correlated with the overexpression of SIRT1.

The major difference between our result and former studies was that we firstly reported the clinical significance of the cytoplasmic SIRT1 expression as a tumor suppressor. Since molecular experiments confirmed the cytoplasmic SIRT1 enhanced caspases-dependent apoptosis,[20,21] the result of cytoplasmic SIRT1 as a tumor suppressor is reasonable. As the nuclear SIRT1 prevent apoptosis while cytoplasmic SIRT1 enhances apoptosis, the alteration of intracellular SIRT1 localization from nuclei to cytoplasm might be a novel way for HCC therapy. It was reported SIRT1 subcellular localization may change during the development and various physiological and pathological stimuli.[24]

Although mechanisms yet to be further elucidated, Tanno *et al*.[24] reported a SIRT1 nucleo-cytoplasmic shuttling mechanism to participate in differentiation and cell death. However, there is also a possibility that the overexpression of SIRT1 is a consequence, rather than a cause, of tumorigenesis in HCC. More research is needed to explore the complex role of SIRT1 especially the cytoplasm-localized SIRT1 in HCC tumorigenesis.[1,3]

CONCLUSION

SIRT1, P53 and YAP2 were significantly overexpressed and the overexpression of nuclear SIRT1 was positively related to the overexpression of P53 and YAP2. Whether SIRT1 was a tumor promoter or tumor suppressor may depend on the protein location. While the nuclear SIRT1 functions as a tumor promoter and cytoplasmic SIRT1 functions as a tumor suppressor, the role of SIRT1 in HCC should be reconsidered.

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