SIRT1 and c-Myc Promote Liver Tumor Cell Survival and Predict Poor Survival of Human Hepatocellular Carcinomas

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Abstract

The increased expression of SIRT1 has recently been identified in numerous human tumors and a possible correlation with c-Myc oncogene has been proposed. However, it remains unclear whether SIRT1 functions as an oncogene or tumor suppressor. We sought to elucidate the role of SIRT1 in liver cancer under the influence of c-Myc and to determine the prognostic significance of SIRT1 and c-Myc expression in human hepatocellular carcinoma. The effect of either over-expression or knock down of SIRT1 on cell proliferation and survival was evaluated in both mouse and human liver cancer cells. Nicotinamide, an inhibitor of SIRT1, was also evaluated for its effects on liver tumorigenesis. The prognostic significance of the immunohistochemical detection of SIRT1 and c-Myc was evaluated in 154 hepatocellular carcinoma patients. SIRT1 and c-Myc regulate each other via a positive feedback loop and act synergistically to promote hepatocellular proliferation in both mice and human liver tumor cells. Tumor growth was significantly inhibited by nicotinamide in vivo and in vitro. In human hepatocellular carcinoma, SIRT1 expression positively correlated with c-Myc, Ki67 and p53 expression, as well as high a-fetoprotein level. Moreover, the expression of SIRT1, c-Myc and p53 were independent prognostic indicators of hepatocellular carcinoma. In conclusion, this study demonstrates that SIRT1 expression supports liver tumorigenesis and is closely correlated with oncogenic c-MYC expression. In addition, both SIRT1 and c-Myc may be useful prognostic indicators of hepatocellular carcinoma and SIRT1 targeted therapy may be beneficial in the treatment of hepatocellular carcinoma.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most frequent cause of cancer mortality worldwide [1]. Genetic alterations in HCC have been extensively studied yielding the identification of broad molecular categories of HCC [2]. Among numerous potential oncogenic pathways, c-Myc has been observed to be a potent initiating oncogene of liver tumors and inactivation of c-Myc is sufficient to induce sustained regression of MYC-initiated liver tumors in mice [3]. Intriguingly, c-Myc activates the tumor suppressor p53, therefore, additional regulatory mechanisms that are closely related with the oncogenic potential of c-Myc and involve the inactivation of p53 could be essential. Among the direct inhibitors of the p53 protein, SIRT1 is emphasized for its deacetylation activity [4,5]. In addition, a positive feedback loop between c-Myc and SIRT1 during tumorigenesis would imply a predominant oncogene function for SIRT1 [6,7]. Conversely, a tumor suppressive role for SIRT1 is suggested by a reciprocal transcriptional control mechanism between c-Myc and SIRT1 [8]. Thus, the role of SIRT1 in human tumors with oncogenic MYC expression remains controversial. Overall, independent of MYC, the deacetylation mediated inhibition of several tumor suppressors including FoxO3 [9], Rb [10], and Ki70 [11], together suggest that SIRT1 has significant tumor promoting activity [12,13]. Moreover, recent reports have shown that the expression of SIRT1 is associated with a poor prognosis in specific human tumors including hepatocellular carcinoma [14], gastric cancer [15], breast cancer [16], and diffuse large B cell lymphoma [17]. SIRT1 expression has additionally been implicated as a contributing mechanism for increased resistance to anticancer agents [18,19]. However, there are additional conflicting reports regarding the tumor suppressing capability of SIRT1 [8,20,21]. In ovarian cancer patients, SIRT1 expression predicts a favorable prognosis despite high expression in malignant tumors compared with benign or borderline tumors [22]. In colon cancer, SIRT1 was found to negatively regulate the
expression between SIRT1 and c-Myc. As evidence of the effect of the functional knock-down of SIRT1, a significant increase in acetylated p53 and p21 was also detected (Figure 2 C). Specifically, with SIRT1 overexpression of c-Myc whereas the shRNA mediated knock down of SIRT1 resulted in decreased c-Myc expression (Figure 3 A and B). Moreover, knock-down of SIRT1 resulted in a detectable increase in acetylated p53 (Figure 3 A). Specifically, with SIRT1 overexpression, cells were more proliferative than control cells and the proliferation of cells subjected to SIRT1 knock-down decreased compared with control cells (Figure 3 C). Furthermore, the chemical inhibitor NAM decreased the proliferation and colony forming capacity of both human HCC cell lines (Figure 3 D).

**SIRT1 Expression in c-Myc and DDC Induced Liver Tumors and the Effect of SIRT1 Inhibition on Liver Tumor Development in Mice**

As previously reported [24], the non-oncogenic hepatotoxin 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) along with c-Myc overexpression in a mouse model reproducibly results in uniform liver tumor development within four weeks of initiation. We sought to exploit the predictable kinetics of the MYC/DDC tumor model system to determine the relationship of MYC and SIRT1 in vivo. Seven days after induction of c-Myc overexpression in conjunction with DDC treatment, small proliferating atypical cell foci appeared (Figure 4 A); 21 days after induction of MYC-DDC, coalescing tumor cells form grossly identifiable large tumor nests (Figure 4 A). Tumor cell volumes were noted to be small with increased nuclear to cytoplasmic ratios, and with prominent nucleoli. Frequent mitoses were also identifiable (Figure 4 A).

Since the expression of SIRT1 strongly correlated with c-Myc expression in the Tet-O-MYC cells, we examined the expression of c-Myc and SIRT1 in MYC-DDC tumors (Figure 4 B). In control liver, c-Myc and SIRT1 was not detectable in normal hepatocytes. However, expression of c-Myc and SIRT1 expression was significantly increased in hepatic tumors in MYC-DDC mouse liver. Specifically, tumor cells demonstrated stronger expression of c-Myc and SIRT1 than adjacent non-transformed hepatocytes (Figure 4 B). Next, we investigated the effect of SIRT1 inhibition on liver tumorigenesis in vivo. In the control group, all five MYC-DDC mice without treatment by NAM developed liver tumors at four weeks. Conversely, the incidence of tumor development in the liver of MYC-DDC mice was significantly attenuated by treatment with NAM (P = .038) (Figure 4 C). Only three of seven NAM
treated MYC-DDC mice developed liver tumors. Two of three had grossly detectable liver tumors and one mouse had only microscopically detectable tumor. Between the control and NAM treated groups, there was no difference in either body or liver weight (Figure 4 D). Histologically, there was no evidence of NAM related toxicity in the liver of NAM treated mice. NAM treatment in MYC-DDC mice resulted in increased acetylation of the tumor suppressor protein p53 (Figure 4 E).

Association of SIRT1 and c-Myc Expression with Clinicopathologic Characteristics of HCC Patients

Next, in order to investigate the potential clinical relevance of these findings, we undertook an examination of c-Myc, SIRT1, p53 and Ki67 expression in a cohort of human HCC tissues. As demonstrated in Figure 5, immunoreactivity for c-Myc, SIRT1, p53 and Ki67 was found primarily in the nuclei of tumor cells. Positive expression of SIRT1, c-Myc, p53 and Ki67 was seen in 36% (55/154), 47% (72/154), 18% (27/154), and 38% (58/154) of patients, respectively (Table 1). The expression of SIRT1 was significantly associated with preoperative serum a-fetoprotein (AFP) levels (P=0.032), presence of HBV infection (P=0.023), and the expression of c-Myc (P=0.014), p53 (P<0.001) and Ki67 (P=0.011). The correlation coefficient between c-Myc and SIRT1 expression was 0.190 (P=0.014). The expression of c-Myc significantly correlated with preoperative serum albumin levels (P=0.011) and the TNM stage (P=0.02). The expression of p53 also significantly associated with serum AFP level (P=0.036), HBV infection (P=0.002), and serum bilirubin level (P=0.03). The expression of Ki67 significantly associated with serum AFP level (P=0.005), HBV infection (P=0.03), and high histologic grade (P=0.04). Additional variables detailed in Table 1 displayed no statistically significant association with the expression of SIRT1, c-Myc, p53, or Ki67 in HCCs.

Expression of SIRT1 and c-Myc Predicts Shorter Survival of HCC Patients

We next sought to determine the relationship if any of oncoprotein SIRT1, c-Myc, p53, and Ki67 to OS and DFS as shown in Table 2. Advanced tumor stage (P=0.001), serum AFP level (P=0.008), serum albumin level (P=0.029), as well as the expression of SIRT1 (P<0.001), c-Myc (P=0.001), and p53 (P=0.001) were all significantly associated with shorter OS according to univariate analysis Advanced tumor stage (P=0.001), serum AFP level (P=0.001), serum albumin level (P=0.017), high histologic grade (P=0.014) and expression of SIRT1 (P<0.009), c-Myc (P=0.011), and p53 (P=0.006) were associated with significantly shorter DFS according to univariate analysis (Figure 5 B).

Next, multivariate analysis was performed using all patients with complete information for all variables (Table 3). Variables significantly associated with OS and DFS upon univariate Cox regression analysis were included in the multivariate analysis. The subjects with SIRT1 expression had a 2.144-fold (95% CI, 1.265–3.635) greater risk of death (P=0.005) and the patients with c-Myc expression had a 2.902-fold (95% CI, 1.598–5.269) greater risk of death (P<0.001). The expression of c-Myc was also predictive of reduced DFS with a Pvalue of 0.006 (hazard ratio, 1.851; 95% CI, 1.19–2.879). SIRT1 expression was not associated with DFS according to multivariate analysis (P=0.474). Tumor stage, serum albumin level, and p53 expression were also independent prognostic factors significantly associated with OS and DFS according to multivariate analysis.

Discussion

In the present study the role of SIRT1 in liver cancer was investigated. Studies included an examination of the biologic effect of SIRT1 on cell proliferation and survival as well as an analysis of SIRT1 as a possible prognostic factor in human HCC. We utilized a conditional transgenic mouse and cell line for the controlled expression of c-Myc to investigate the role of SIRT1 expression in c-Myc driven liver tumorigenesis. We also investigated the possible clinical significance of our findings in a cohort of human HCC tumor samples. Of significance, we report that: (1) SIRT1 expression is tightly correlated with oncogenic (c-Myc) expression and promotes the proliferation of liver cancer cells; (2) genetic or physio-chemical inhibition of SIRT1 with shRNA to SIRT1 or NAM respectively, inhibits the proliferation of liver cancer cells; (3) SIRT1 inhibition in vivo with NAM significantly reduces tumorigenesis in a mouse tumor model; (4) SIRT1 expression is significantly associated with the expression of c-Myc, p53, and Ki67; and (4) the expression of SIRT1 and c-Myc is associated with a significantly shortened survival in HCC patients. Taken together, our findings suggest that SIRT1 cooperates with c-Myc for the progression of HCCs and influences the prognosis of HCC patients.

The role of SIRT1 in tumorigenesis is controversial especially in the association between SIRT1 and oncogenic c-Myc. Recently, positive correlation between c-Myc and SIRT1 in tumorigenesis has been reported [6,7]. In colorectal cancer, c-Myc increased both SIRT1 protein level and deacetylase activity of SIRT1 [7]. In addition, SIRT1 also increased transcriptional activity of c-Myc and its stabilization in fibroblasts [6,7]. SIRT1 mediated deacetylation of c-Myc increased the half-life of c-Myc and promoted c-Myc/Max association that resulted in suppression of cellular senescence and inhibition of apoptosis [6,7]. In contrast, one early report has shown that SIRT1 de-stabilized c-Myc by deacetylation despite increasing c-Myc transcription and therefore suggesting SIRT1 as a negative regulator of Myc directed tumorigenesis [8]. In the present study, we demonstrate that the expression of SIRT1 and c-Myc are positively correlated and both promote the proliferation of tumor cells. Induced c-Myc expression in Tet-O-MYC cells results in a corresponding increased expression of SIRT1 and overexpression of SIRT1 also produced increased c-Myc expression. Conversely, oncogenic c-Myc silence-
performed in the presence of 10 mM NAM. A colony-forming assay was performed by culture of 2×10^3 cells for two to three days. Colony forming assay were performed in the presence of 10 mM NAM. A colony-forming assay was performed by culture of 2×10^3 cells for 7 days in 24-well culture plates. A single asterisk indicates P<0.05, two asterisks indicate P<0.001.

Confirming these findings, MYC driven tumors in vivo demonstrate high numbers of both mitotic and apoptotic cells (Figure S1 C). Conversely, the expression of cleaved caspase-3 decreased with SIRT1 over-expression (Figure S1 D). Therefore, SIRT1 over-expression may also promote cell survival through the inhibition of p53-mediated apoptosis. Our results also demonstrate that knock down of SIRT1 in liver cancer cells induces a detectable increase in acetylated p53 along with an overall decrease in the proliferative activity of cells. Moreover, increased acetylation of p53 in NAM treated MYC-DDC mice in vivo were accompanied by decreased hepatocellular tumorigenesis. Accordingly, our findings suggest that SIRT1 may contribute to liver tumorigenesis at least in part by controlling cellular proliferation and survival through the modulation of p53 activity. These observations also support and may provide a plausible contributing mechanism for our observation that patients with SIRT1 expressing tumors possess an unfavorable prognosis. However, the role of SIRT1 in liver tumorigenesis remains incompletely understood given the numerous biologic roles that have been described for the sirtuins. Moreover, the relationship between SIRT1 and p53 remains complex since the mutational status of p53 may alter the biologic influence of SIRT1 on p53 activity. As we have shown in Figure 3, the knockdown of SIRT1 in Huh7 cells showed more effect on suppression of c-Myc mRNA than HepG2 cells. However, the effect of SIRT1 knockdown in Huh7 cell has a smaller effect on cell proliferation compared to the effect in HepG2 cells. This discrepancy may relate to the mutational status of p53 in Huh7 and HepG2 cells. Huh7 cells [28] are mutant for p53 and HepG2 cells [29] are wild type for p53. In Huh7 cells, suppression of c-Myc by SIRT1 knockdown may induce inhibition of cell proliferation. However, the influence of SIRT1 knockdown to mutated p53 of Huh7 cell is unpredictable despite our demonstration that of SIRT1 knockdown increases p53 acetylation in Huh7 cells. Therefore, we propose that the combined effects of SIRT1 knockdown on c-Myc and mutant p53 likely results in a diminished effect on the proliferation of Huh7 cells. In contrast, the inhibitory effect of SIRT1 knockdown on HepG2 cells may relate primarily to the inhibition of wild type p53 by deacetylation despite limited measurable effect on c-Myc expression. Additional studies beyond the scope of this work are needed to clarify the exact role of SIRT1 relative to p53 mutational status.

The role of SIRT1 in tumorigenesis is controversial. Our study has shown that SIRT1 acts primarily as a tumor promoting factor in hepatocellular carcinogenesis. Together our data demonstrate at least three possible mechanisms to account for this tumor promoting activity. Our clinical sample analysis suggests a strong positive correlation between SIRT1 and oncogenic MYC. Moreover, we have provided in vitro evidence that SIRT1 promotes oncogenic c-MYC expression simultaneously with a suppressive effect of oncogene induced apoptosis through the p53 axis. However, an additional potential mechanistic explanation for SIRT1 tumor promoting activity may involve the activation of oncogenes in addition to c-Myc. In contrast to previous reports that SIRT1 negatively regulates oncoprotein a-catenin in colon cancer [21] and survivin in breast cancer [30], in our studies SIRT1 overexpression induced increased a-catenin mRNA and protein expression, increased a-catenin TCF/LEF transcription co-activation and increased expression of a-catenin target genes cyclin D1 and survivin in liver tumor cells (Figure S2). Therefore, we propose that the combined effects of increased SIRT1 signaling on various oncogenes such as c-Myc and a-catenin, is multifactorial and at least in part is protection against apoptosis involving survivin and p53 during hepatic tumorigenesis.

In order to begin to investigate whether SIRT1 activity could be manipulated to an observable effect in vivo, we undertook a series of experiments to inhibit SIRT1 activity using its physiologic inhibitor NAM. Accordingly, in a tumor prevention approach, we found that the administration of NAM in DDC treated c-Myc transgenic mice significantly decreased the incidence of liver tumorigenesis. In addition to the role of NAM as a SIRT1 inhibitor, NAM may also function as an antioxidant. Therefore, in order to account for the possibility that NAM was exerting a predominant antioxidant effect on liver tumorigenesis as had been previously described, we tested the effect of the established antioxidant N-acetyl-L-cysteine (NAC) on liver tumorigenesis. We administered NAC (10 mg/mL) in the drinking water to four MYC-DDC mice. We observed that NAC did not noticeably influence liver tumorigenesis as all four NAC treated mice developed liver tumors (Figure S3). Consequently, we conclude the antitumor effects of NAM are likely independent of an anti-
SIRT1 and c-Myc in Hepatocellular Carcinoma

A

Huh7

|       | CV | SIRT1 | CV | shRNA |
|-------|----|-------|----|-------|
| SIRT1 |    |       |    |       |
| c-Myc |    |       |    |       |
| p53   |    |       |    |       |
| ac-p53|    |       |    |       |
| actin |    |       |    |       |

HepG2

|       | CV | SIRT1 | CV | shRNA |
|-------|----|-------|----|-------|
| SIRT1 |    |       |    |       |
| c-Myc |    |       |    |       |
| p53   |    |       |    |       |
| ac-p53|    |       |    |       |
| actin |    |       |    |       |

B

Huh7

|       | control vector | SIRT1 overexpression |
|-------|----------------|----------------------|
| SIRT1 | 0.0 | 10.0 *** |
| c-Myc | 0.0 | 0.5 ** |

HepG2

|       | control vector | SIRT1 overexpression |
|-------|----------------|----------------------|
| SIRT1 | 0.0 | 1.5 ** |
| c-Myc | 0.0 | 1.0 *** |

C

|       | control vector | SIRT1 overexpression | shRNA for SIRT1 |
|-------|----------------|----------------------|-----------------|
| Huh7  | 50.0 **        | 100.0 **             | 75.0 **         |
| HepG2 | 50.0 **        | 100.0 **             | 75.0 **         |

D

|       | Control | NAM 10 mM |
|-------|---------|-----------|
| Huh7  | 80.0 ** | 60.0 **   |
| HepG2 | 80.0 ** | 60.0 **   |

Huh7

|       | CV | SIRT1 | CV | shRNA |
|-------|----|-------|----|-------|
|       |    |       |    |       |
|       |    |       |    |       |

HepG2

|       | CV | SIRT1 | CV | shRNA |
|-------|----|-------|----|-------|
|       |    |       |    |       |
|       |    |       |    |       |
oxidant function and therefore more likely correlate with the inhibitory effects of SIRT1.

In the course of our studies, we were also interested in extending our observations to an established cohort of archived human HCC tumor samples. SIRT1 expression previously reported between 31% and 56% of HCC [14,29,31], and in this study 36% of HCC showed SIRT1 expression. In line with the positive correlation between c-Myc and SIRT1 expression in Tet-O-MYC liver cancer cells, we detected a significant immunohistochemical relationship between SIRT1 and c-Myc in human HCCs. Moreover, the expression of SIRT1 and c-Myc predicted shorter OS and DFS of HCC patients. Together these findings suggest that SIRT1 appears to possess an important role in liver tumorigenesis. In addition, c-Myc expression revealed a significant relationship with established unfavorable clinicopathologic features of HCC patients including low serum albumin and advanced clinical stage. Moreover, when we analyzed the prognostic impact of c-Myc and SIRT1 for HCC patients with the combination of these two molecules, patients expressing both c-Myc and SIRT1 displayed shorter OS and DFS compared with the patients that did not express both c-Myc and SIRT1 (mean overall survival time; 48.45±7.28, 115.08±7.66 months, respectively) (Figure S4). There was also a population of tumors expressing either c-Myc or SIRT1 (c-Myc+/SIRT1+ or c-Myc+/SIRT1−) and these subjects showed a poorer prognosis than the c-Myc-/SIRT1− group. As demonstrated in Figure S4, the survival curve of c-Myc-/SIRT1+ or c-Myc+/SIRT1− groups fell between the c-Myc+/SIRT1+ and c-Myc-/SIRT1− groups. Ten-year survival rate of c-Myc-/SIRT1−, c-Myc+/SIRT1+, c-Myc+/SIRT1−, and c-Myc+/SIRT1+ groups were 70%, 25%, 31%, and 14%, respectively. Therefore, these data suggest there is a correlation between c-Myc and SIRT1 in hepatic tumorigenesis as well as an independent role of c-Myc and SIRT1 in hepatic tumorigenesis. Similar to our results, it has previously been reported that c-Myc expression correlates with the progression and poor prognosis of HCC [32,33]. However, still it is not clear whether SIRT1 is real prognostic indicator of HCC or not. One recent report suggested SIRT1 as a possible prognostic indicator of HCC [14] but other report could not find prognostic significance of SIRT1 despite higher expression of SIRT1 in cancer tissue compared to adjacent normal tissue [31].

From a clinicopathologic perspective, in this study we also observed that SIRT1 expression significantly correlated with preoperative AFP levels. The serum level of AFP in SIRT1 positive patients (mean ± standard error, 4336±1890) was markedly higher than in SIRT1 negative patients (mean ± standard error, 987±205) (t-test, 2-tailed, P = 0.027). This positive correlation between SIRT1 and serum AFP levels further supports a likely role for SIRT1 in HCC development and progression. Interestingly, SIRT1 expression also significantly correlated with HBV infection. When considering the previous reports that HBV X protein is involved in liver tumorigenesis by inducing the expression of histone deacetylase 1 [34], it raises the intriguing possibility that SIRT1 is also contributing to liver tumorigenesis through its activity as a histone deacetylase induced in HBV infected patients. Further study beyond the scope of this work is warranted to further define the precise tumor promoting mechanism provided by SIRT1.

In summation, our results suggest that SIRT1 cooperates with c-Myc in liver tumorigenesis and may be an important molecular target for modulating liver cancer. As previously suggested by others [33–36], in this study we also find that inhibition of SIRT1 may be an effective strategy for the prevention of tumor development in high-risk patients or as a therapeutic strategy in established tumors. In conclusion, our findings indicate that SIRT1 and c-Myc may be involved in the progression of HCCs and both of these molecules may be useful as clinical indicators of overall HCC prognosis.

Materials and Methods

Ethics

All animal experiments were performed in accordance with a protocol approved by the Stanford University’s Administrative Panels on Laboratory Animal Care. This study was conducted with institutional review board approval. Informed consent was provided according to the Declaration of Helsinki.

Transgenic Mice and Cell Lines

In this study we utilized bitransgenic mice that conditionally express the c-MYC proto-oncogene in hepatocytes [3,39]. c-MYC expression is activated by removing doxycycline treatment (100 μg/ml) from the drinking water of mice. To investigate the role of SIRT1 under the influence of c-Myc at zero, we utilized a primary culture tumor cell line generated from the liver tumors of Tet-O-MYC mice [3,39]. The human HCC cell lines Huh7 and HepG2 were also utilized. All cell lines were cultured in Dulbecco’s minimal essential medium. Nicotinamide (NAM; Sigma Aldrich, St. Louis, MO) was dissolved in distilled water.

Genotyping with Polymerase Chain Reaction

DNA was isolated from the tail of the mice or Tet-O-MYC cells using the Qiagen DNeasy kit (Qiagen, Valencia, CA) in accordance with the manufacturer’s directions. The LAP-tTa segment was detected using the following primers: LAP-tTa forward 5¢-GCTGCTTAATGAGGTCGG-3¢ and LAP-tTa reverse 5¢-CTCTGCACCTTGTTTGATCT-3¢. The Tet-O-Myc construct was detected with the following primers: c-Myc forward 5¢-CCTACCCCTCAGAGGAGC-3¢ and c-Myc reverse 5¢-CTCTGACCTTTTGCAAGAG-3¢. DNA was amplified using the following polymerase chain reaction (PCR) protocol: 94°C denaturation for 2 minutes followed by 33 cycles of 94°C for 15 seconds, 59°C annealing for 30 seconds, and 72°C for 30 seconds, followed by a 5 minute extension at 72°C. PCR products were resolved on a 1.5% gel.
A) Tet-O-MYC mice with MYC-ON and DDC treatment

B) Control vs. MYC-DDC, 4 weeks

C) NAM - vs. NAM +

D) BW, LW, LI (%)

E) NAM treatment
Tumorigenecity Assays

To accelerate liver tumorigenesis in Tet-O-MYC mice, we fed a diet containing 0.1% DDC (Sigma Aldrich, St. Louis, Missouri, United States) in Tet-O-MYC mice as previously described (MYC-DDC mice).\(^2\) Five MYC-DDC mice were sacrificed at 7, 14, 17, 21, and 28 days after being fed a DDC enriched diet with c-Myc activation by removing doxycycline from the drinking water. An additional twelve MYC-DDC mice were used for a determination of the effect of NAM on liver tumorigenesis in MYC-DDC mice. Seven MYC-DDC mice were administrated NAM by adding 10 mg/mL NAM into the drinking water and five mice did not receive NAM. Thereafter, mice were sacrificed at four weeks and evaluated for tumor development. Liver tissues were snap frozen in liquid nitrogen or were prepared for histology by fixing in 10% buffered formalin and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin.

Transfection of SIRT1 Plasmid DNA and SIRT1 Short Hairpin RNA Plasmid

Human SIRT1 plasmid DNA (pDNA) (Addgene, plasmid 1769, Cambridge, MA) was cloned into pCMV-tag2B (Stratagene, La Jolla, CA). The same plasmid without the SIRT1 DNA insert was used for transfection.

Table 1. Clinicopathologic variables and the expression status of SIRT1, c-Myc, p53, and Ki67.

| Characteristics | SIRT1 | c-Myc | p53 | Ki67 |
|----------------|-------|-------|-----|------|
| Sex Male       | 132   | 47 (36%) | 0.945 | 64 (48%) | 0.291 | 20 (15%) | 0.057 | 51 (39%) | 0.541 |
| Female         | 22    | 8 (36%)  | 0.593 | 31 (47%) | 0.963 | 13 (20%) | 0.541 | 24 (36%) | 0.773 |
| Age, y <55     | 66    | 22 (33%) | 0.593 | 31 (47%) | 0.963 | 13 (20%) | 0.541 | 24 (36%) | 0.773 |
| ≥55            | 88    | 33 (38%) | 0.828 | 33 (48%) | 0.81  | 7 (10%)  | 0.03  | 27 (39%) | 0.735 |
| AFP, ng/mL <100| 101   | 30 (30%) | 0.032 | 45 (45%) | 0.45  | 13 (13%) | 0.036 | 30 (30%) | 0.005 |
| ≥100           | 53    | 25 (47%) | 0.516 | 27 (51%) | 0.291 | 14 (26%) | 0.735 | 28 (33%) | 0.43   |
| HBV Negative   | 42    | 9 (21%)  | 0.023 | 20 (48%) | 0.895 | 1 (2%)   | 0.002 | 10 (24%) | 0.03   |
| Positive       | 112   | 46 (41%) | 0.677 | 52 (46%) | 0.677 | 26 (23%) | 0.48   | 48 (43%) | 0.37   |
| HCV Negative   | 144   | 52 (36%) | 0.677 | 70 (49%) | 0.08  | 27 (19%) | 0.132 | 54 (38%) | 0.875 |
| Positive       | 10    | 3 (30%)  |       | 2 (20%) |       | 0 (0%)  |       | 4 (40%)  |       |
| Liver cirrhosis Absence | 81    | 31 (38%) | 0.485 | 42 (52%) | 0.182 | 15 (19%) | 0.735 | 33 (41%) | 0.406 |
| Presence       | 73    | 24 (33%) |       | 30 (41%) |       | 12 (16%) |       | 25 (34%) |       |
| Bilirubin, mg/dl<0.7 | 69    | 24 (35%) | 0.828 | 33 (48%) | 0.81  | 7 (10%)  | 0.03  | 27 (39%) | 0.735 |
| ≥0.7           | 85    | 31 (36%) |       | 39 (46%) |       | 20 (24%) |       | 31 (36%) |       |
| Albumin, ng/dl<3.5 | 17    | 5 (29%)  | 0.565 | 3 (18%)  | 0.011 | 2 (12%)  | 0.507 | 8 (47%)  | 0.397 |
| ≥3.5           | 137   | 50 (36%) |       | 69 (50%) |       | 25 (18%) |       | 50 (36%) |       |
| TNM stage I    | 60    | 21 (35%) | 0.839 | 26 (43%) | 0.02  | 8 (13%)  | 0.506 | 19 (32%) | 0.329 |
| II              | 58    | 22 (38%) |       | 22 (38%) |       | 11 (19%) |       | 26 (45%) |       |
| III & IV       | 36    | 12 (33%) |       | 24 (67%) |       | 8 (22%)  |       | 13 (36%) |       |
| Histologic grade Low | 93    | 31 (33%) | 0.446 | 47 (51%) | 0.245 | 12 (13%) | 0.062 | 29 (31%) | 0.04   |
| High           | 61    | 24 (39%) |       | 25 (41%) |       | 15 (25%) |       | 29 (48%) |       |
| Ki67 Negative  | 96    | 27 (28%) | <0.001 | 47 (49%) | 0.48  | 13 (14%) | 0.094 |       |       |
| Positive       | 58    | 28 (48%) |       | 25 (43%) |       | 14 (24%) |       |       |       |
| p53 Negative   | 127   | 37 (29%) |       | 59 (46%) | 0.873 |       |       |       |       |
| Positive       | 27    | 18 (67%) |       | 13 (48%) |       |       |       |       |       |
| c-Myc Negative | 82    | 22 (27%) | 0.014 |       |       |       |       |       |       |
| Positive       | 72    | 33 (46%) |       |       |       |       |       |       |       |

Abbreviations: AFP, α-fetoprotein.
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Figure 4. SIRT1 expression in liver tumors and the inhibition of SIRT1 decreases hepatic tumorigenesis in mice. A) Seven days after induction of c-Myc overexpression along with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) treatment, small atypical cell foci appear and tumors nests gradually increase in size. B) Immunohistochemical staining for c-Myc and SIRT1 shows absent expression of c-Myc and SIRT1 in hepatocytes of control mice without c-Myc activation. In parallel with the development of hepatic tumors in MYC-DDC mice, the expression of c-Myc and SIRT1 increase in tumor cells. Tumor cells demonstrate strong expression of c-Myc and SIRT1 in comparison to adjacent non-transformed hepatocytes. C) All five MYC-DDC mice without nicotinamide (NAM) treatment developed grossly visible multinodular hepatic tumors. Conversely, only three of seven MYC-DDC mice treated with NAM (10 mg/mL) developed hepatic tumor (P = 0.038). D) Body weight (BW), liver weight (LW), and liver index (LI; liver weight/body weight x100) in DDC treated c-Myc transgenic mice (MYC-DDC mice) with or without administration of NAM. There is no difference of BW, LW, and LI between control and NAM administrated MYC-DDC mice. E) Administration of NAM resulted in a detectable increase in acetylation of p53 protein.
used as a control vector (CV). Plasmid for SIRT1 short hairpin RNA (shRNA) and CV was kindly provided by A. Brunet (Stanford University, Stanford, CA). All plasmids were amplified in DH5α Escherichia coli competent cells (Invitrogen, Carlsbad, CA) and were purified using an endo-free plasmid mega-prep kit (Qiagen, Valencia, CA). Transient transfection of Tet-O-MYC, Huh7 and HepG2 cells with human SIRT1 pDNA and shRNA was performed using Lipofectamin 2000 (Invitrogen, Carlsbad, CA). 44 hours after transfection, the cells were harvested and used for further experiments.

Cell Proliferation Assay

The number of live cells was counted by using trypan blue exclusion. For the assessment of cell proliferation and survival/viability, a commercially available 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (Roche Applied Science, Indianapolis, IN) and colony-forming exclusion. For the assessment of cell proliferation and survival/viability, a commercially available 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (Roche Applied Science, Indianapolis, IN) and colony-forming exclusion. For the assessment of cell proliferation and survival/viability, a commercially available 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (Roche Applied Science, Indianapolis, IN) and colony-forming exclusion. For the assessment of cell proliferation and survival/viability, a commercially available 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (Roche Applied Science, Indianapolis, IN) and colony-forming exclusion.

Quantitative Reverse-transcription Polymerase Chain Reaction

RNA isolation was performed with the RNeasy Mini Kit (Qiagen Sciences, Valencia, CA). After DNase treatment, reverse transcription of 1.5 μg RNA was performed with Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out using the Applied Biosystems Prism 7900HT Sequence Detection System and Sybr Green or Taqman PCR Master Mix (Applied Biosystems, Foster City, CA). All experiments were performed in triplicate and the results were normalized to the expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference housekeeping gene. Primer sequences for PCR were: c-Myc forward 5′-CTGGGAGGAGGAGGAGGACT-3′, reverse 5′-GGGACGACCTGGAATTCTCT-3′; SIRT1 forward 5′-TGCTGGCTTAATAGTGTTGGA-3′, reverse 5′-CTGAGGGCAGGAAGGAAAGTGT-3′; α-catenin forward 5′-GTCAGCCTGTTCTCCTGTGAA-3′, reverse 5′-GATCTGCTATCCCTGTACTA-3′; cyclin D1 forward 5′-TGGACCCCTGTAAGAGAGG-3′, reverse 5′-AAGTGCGTTGTGCGGTAGC-3′; survivin forward 5′-GGAGCAGCCGATCTCTACAT-3′, reverse 5′-GGCACTTTCTTCGAGATTCC-3′; GAPDH forward 5′-GAGGCCGATCTTCCTGTG-3′, reverse 5′-CACACC-GACGTTCACATT4T-3′.

Luciferase Reporter Assay

Dual-Light Reporter Gene Assay (Applied Biosystems, Foster City, CA) was performed using the pMegaTOPFLASH, pMegaFOPFLASH, 5× HRE, or LacZ plasmids as previously described [40–42].

Western Blot Analysis

Total proteins lysed with RIPA buffer were separated by SDS-PAGE and transferred to a PVDF membrane. The blot was probed with primary antibodies for c-Myc (Santa Cruz, clone C-19, CA), SIRT1 (Santa Cruz, clone H-300, CA), p53 (Santa Cruz, clone FL-393, CA), acetyl-p53 (Santa Cruz, clone FL-393, CA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz, clone H-89, CA), survivin (Santa Cruz, clone H-89, CA), α-catenin (BD Transduction Laboratories, Bedford, MA), cyclin D1 (Epitomics, Burlingame, CA) and histone H3 (Abcam, Cambridge, MA). Blots were then incubated with secondary antibodies conjugated to horseradish peroxidase before detection with enhanced chemiluminescence (ECL) substrate.

Table 2. Univariate Cox regression analysis for overall survival and disease-free survival in hepatocellular carcinoma patients.

| Characteristics          | No. | OS HR | 95% CI | P   | DFS HR | 95% CI | P   |
|--------------------------|-----|-------|--------|-----|--------|--------|-----|
| Age ≥55 y (vs <55 y)    | 88/154 | 1.311 | 0.787–2.183 | 0.298 | 1.284 | 0.853–1.931 | 0.231 |
| Sex female (vs male)     | 22/154 | 0.861 | 0.410–1.807 | 0.692 | 0.97 | 0.540–1.743 | 0.92 |
| HBV positive (vs negative) | 112/154 | 1.111 | 0.631–1.957 | 0.716 | 1.221 | 0.764–1.951 | 0.404 |
| HCV positive (vs negative) | 3/154 | 1.451 | 0.582–3.619 | 0.425 | 1.838 | 0.888–3.803 | 0.101 |
| AFP ≥100 ng/ml (vs <100 ng/ml) | 53/154 | 1.952 | 1.192–3.196 | 0.008 | 1.968 | 1.317–2.943 | 0.001 |
| Albumin <3.5 mg/dl (vs ≥3.5 mg/dl) | 17/154 | 2.129 | 1.081–4.190 | 0.029 | 2.05 | 1.139–3.690 | 0.017 |
| Bilirubin ≥0.7 mg/dl (vs <0.7 mg/dl) | 85/154 | 1.274 | 0.775–2.095 | 0.339 | 1.078 | 0.722–1.611 | 0.712 |
| Stage I                  | 60/154 | 0.001 | 0.000–1.000 | 0.001 | 0.001 | 0.000–1.000 | 0.001 |
| II                       | 58/154 | 1.657 | 0.879–3.123 | 0.118 | 1.278 | 0.784–2.082 | 0.325 |
| III & IV                 | 36/154 | 3.134 | 1.689–5.816 | <0.001 | 2.49 | 1.516–4.090 | <0.001 |
| Cirrhosis presence (vs absence) | 73/154 | 1.013 | 0.620–1.655 | 0.958 | 0.928 | 0.622–1.384 | 0.714 |
| Histologic grade high (vs low) | 61/154 | 1.598 | 0.970–2.631 | 0.066 | 1.654 | 1.105–2.476 | 0.014 |
| SIRT1 positive (vs negative) | 55/154 | 2.518 | 1.536–4.128 | <0.001 | 1.532 | 1.023–2.294 | 0.039 |
| c-Myc positive (vs negative) | 72/154 | 2.354 | 1.397–3.969 | 0.001 | 1.692 | 1.130–2.533 | 0.011 |
| p53 positive (vs negative) | 27/154 | 2.501 | 1.427–4.384 | 0.001 | 1.97 | 1.219–3.184 | 0.006 |
| KI67 positive (vs negative) | 58/154 | 0.976 | 0.588–1.619 | 0.924 | 1.21 | 0.807–1.815 | 0.355 |

Abbreviations: OS, overall survival; DFS, disease-free survival; AFP, α-fetoprotein; HR, hazard ratio.

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Figure 5. Kaplan-Meier survival analysis in hepatocellular carcinoma. A) Immunohistochemical expression of c-Myc, SIRT1, p53 and K67 in human hepatocellular carcinoma tissue (original magnification ×400). B) Overall survival (OS) and disease-free survival (DFS) in 154 patients according to the TNM stage, c-Myc expression, SIRT1 expression, and p53 expression. doi:10.1371/journal.pone.0045119.g005
ogy, Beverly, MA), acetyl-p53 (Lys379) (Cell Signaling Technology, Beverly, MA), p21 (Santa Cruz, clone M-19, CA), and β-actin (Abcam, Cambridge, MA).

Hepatocellular Carcinoma Patients and Tissue Samples
154 cases of HCC patients who underwent radical resection in Chonbuk National University Hospital between January 1998 and August 2009, and for whom initial diagnostic paraffin-embedded tissue blocks were available were included in the present study. All of the cases were reviewed and reclassified according to the criteria of the World Health Organization Classification [43]. Pathologic staging was reviewed based on the staging system of the American Joint Committee on Cancer [44]. The patients grouped according to sex, age (≥55 versus <55), clinical stage (I versus II versus III and IV), HBV infection, HCV infection, cirrhosis (absence versus presence), AFP level (≥100 ng/ml versus <100 ng/ml) [43], albumin level (<3.5 g/dl versus ≥3.5 g/dl; normal range, 3.5–5.3 g/dl), bilirubin level (≥0.7 mg/dl versus <0.7 mg/dl), histologic grade [low versus high], and immunohistochemical expression of c-Myc, SIRT1, p53, and Ki67. The cut-off value of bilirubin level was determined by receiver operating characteristic curve analysis at the highest possible likelihood ratio point.

Immunofluorescence and Immunohistochemical Staining
Immunofluorescence staining was performed in cultured cells after fixation with cold methanol, and then incubated with the primary antibody for 1 hour. FITC-conjugated secondary antibody was used. Immunocytochemistry was performed in cultured cells after fixation with cold methanol. Immunohistochemistry was performed in paraffin-embedded blocks. For tissue microarray (TMA) of human HCC, two 3.0 mm sized cores per case were arrayed. The tissue sections were treated with a microwave antigen retrieval procedure in sodium citrate buffer. The following markers were used: c-Myc (Santa Cruz, clone 9E10, CA), SIRT1 (Santa Cruz, clone H-300, CA), Ki67 (DAKO, clone MIB1, Denmark), and p53 (Novoceastra, clone DO-7, Newcastle, UK).

Immunohistochemical Scoring for Tissue Microarray
Immunohistochemical analysis of TMA blocks was performed by three pathologists by consensus without knowledge of the clinicopathological information. Immunostaining for c-Myc and SIRT1 was considered positive if 30% or more of the tumor cells stained with an antibody [15–17,45]. For p53 and Ki67 immunostaining, staining of more than 10% of the cells was regarded as positive.

Statistical Analysis
All experiments were performed three times and representative data are presented. Student’s t test and Pearson’s chi-square test were used for comparison between groups. In the survival analysis, the end point of interest was overall survival (OS) and disease-free survival (DFS). The follow-up end point was the date of the last contact or the date of death through November 2010. OS was calculated as the time from diagnosis to the date of death or last contact. Patients who were alive at last contact were treated as censored for OS analysis. DFS was calculated from the time of diagnosis to the date of first recurrence, latent metastasis, death, or last contact. Patients who were alive at last contact and who had not experienced tumor recurrence or latent metastasis were treated as censored for DFS analysis. Univariate and multivariate Cox proportional hazards regression analyses were performed to estimate the impact of clinicopathologic factors and expression of each marker on OS and DFS. Kaplan-Meier survival curves were constructed to further illustrate the impact of OS and DFS when indicated. SPSS software (version 15.0) was used throughout. P values less than 0.05 were considered statistically significant.

Supporting Information
Figure S1 c-Myc expression and increased apoptosis; SIRT1 expression inhibits apoptosis. A) The expression of cleaved caspase-3 directly correlates with c-MYC in a time dependent manner. When oncogenic c-Myc expression is restored over five days (Re-ON), increased cleaved caspase-3 expression returns. B) MYC-ON cells with increased cleaved caspase-3 by immunocytochemistry. C) The tumor of the MYC-DDC mice shows high numbers of mitosis (empty arrows) and apoptosis (arrow heads) (original magnification x40). D) Over-expression of SIRT1 is associated with a decrease in cleaved caspase-3 expression. (TIF)

Figure S2 Over-expression of SIRT1 induces ß-catenin expression. A) Over-expression of SIRT1 in Tet-O-MYC cells increases β-catenin/TCF reporter activity. B) Quantitative real time PCR.
After transfection of Tet-O-MYC cells with a SIRT1 over-expression plasmid, the expression of mRNA for SIRT1, β-catenin, cyclin D1, and survivin significantly increases compared with control vector. C) Over-expression of SIRT1 was induced in the HuH7 and HepG2 human hepatocellular carcinoma cell lines. Over-expression of SIRT1 increases the expression of β-catenin protein. One asterisk indicates P<0.05 and two asterisks indicate P<0.001. (TIF)

Figure S3 The effect of N-acetyl-L-cysteine in hepatic tumorigenesis. Administration of N-acetyl-L-cysteine (NAC) in DDC treated c-Myc transgenic mice (MYC-DDC mice) does not influence liver tumorigenesis. All four MYC-DDC mice with NAC (10 mg/mL) and all five MYC-DDC mice without NAC treatment developed hepatic tumors (P = 0.0001). (TIF)

Figure S4 Kaplan-Meier survival analysis in hepatocellular carcinoma according to the combined expression of c-Myc and SIRT1. Overall survival and disease-free survival in 154 patients according to the combined expression of c-Myc and SIRT1 (TIF)

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Author Contributions

Conceived and designed the experiments: KYJ SJN NL GZT DIB HSP WSM DWF KGS. Obtained approval for the animal experiments: GZT KGS. Obtained institutional review board approval: GZT KGS.

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