Molecular and Cellular Pathobiology

Sirtuin 1 Is Upregulated in a Subset of Hepatocellular Carcinomas where It Is Essential for Telomere Maintenance and Tumor Cell Growth

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Abstract

Hepatocellular carcinoma (HCC) is a highly malignant tumor with a poor prognosis. Treatment of HCC is complicated by the fact that the disease is often diagnosed at an advanced stage when it is no longer amenable to curative surgery, and current systemic chemotherapeutics are mostly inefficacious. Sirtuin 1 (SIRT1) is a class III histone deacetylase that is implicated in gene regulations and stress resistance. In this study, we found that SIRT1 is essential for the tumorigenesis of HCC. We showed that although SIRT1 was expressed at very low levels in normal livers, it was overexpressed in HCC cell lines and in a subset of HCC. Tissue microarray analysis of HCC and adjacent nontumoral liver tissues revealed a positive correlation between the expression levels of SIRT1 and advancement in tumor grades. Downregulation of SIRT1 consistently suppressed the proliferation of HCC cells via the induction of cellular senescence or apoptosis. SIRT1 silencing also caused telomere dysfunction—induced foci and nuclear abnormality that were clearly associated with reduced expressions of telomerase reverse transcriptase (TERT), and PTOP, which is a member of the shelterin complex. Ectopic expression of either TERT or PTOP in SIRT1-depleted cells significantly restored cell proliferation. There was also a positive correlation between the level of induction of SIRT1 and PTOP in human HCC. Finally, SIRT1-silencing sensitized HCC cells to doxorubicin treatment. Together, our findings reveal a novel function for SIRT1 in telomere maintenance of HCC, and they rationalize the clinical exploration of SIRT1 inhibitors for HCC therapy.

Cancer Res; 71(12); 4138–49. ©2011 AACR.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third leading cause of cancer death worldwide (1). Epidemiologic evidence suggests a number of environmental factors associated with the development of HCC, including viral hepatitis type B (HBV) and C (HCV) infections, dietary aflatoxin, the male gender, and chronic liver disease (1, 2); however, the molecular mechanisms of HCC pathogenesis remain elusive. Although the cancer can be eradicated by curative surgery, most HCC patients are diagnosed at advanced stages when it is no longer amenable to curative therapies. The prognosis is very poor for patients who have unresectable tumors, with the median survival of approximately 6 months (3). HCC is highly refractory to chemotherapy. Even with the most effective forms of therapy, such as treatment with the anthracycline-based drug, doxorubicin, the response rate has been low (<20%; ref. 4). Therefore, there is an urgent need to understand HCC carcinogenesis at the molecular level, and to identify novel molecular targets for the development of more efficacious therapeutics.

Sirtuins are mammalian homologues of the yeast silent information regulator 2 (SIR2), which are histone deacetylases that utilizes NAD+ as a cofactor for their functions (5). The yeast SIR2 plays a critical role in the extension of life span by repressing genome instability through establishing the transcriptional silencing of the mating-type loci, the ribosomal DNA locus, and the telomeres (6). In mammals, SIR2 is represented by 7 homologues (SIRT1–7), of which SIRT1 is considered to be the human orthologue of SIR2 (7). SIRT1 is a key regulator of energy metabolism by regulating fuel utilization...
according to the energy status of the organism (8). At the cellular level, SIRT1 deacetylates both histones and nonhistone targets including p53, FOXOs, E2F1, and Ku70 (9), and participates in stress response, DNA repair, and apoptosis (7, 10). The deacetylated form of these proteins enhances cell survival by directing the cell to growth arrest for undergoing DNA repair over apoptosis in response to DNA damage. In addition, recent studies showed that SIRT1 interacts with telomeric repeats (11), and plays a role in telomeric maintenance (11, 12) and genomic stability (13). Overexpression of SIRT1 attenuates telomeric shortening associated with aging and augments homologous recombination (11).

The clinical relevance of SIRT1 expression in HCC has not been examined previously. In this study, we found that SIRT1 is overexpressed in a subset of HCC. Reduced SIRT1 expression inhibits proliferation of hepatoma cells. Furthermore, we discovered that SIRT1 plays a role in telomeric maintenance via regulating the expression of telomerase and members of the shelterin complex. Our data suggest that the inhibition of SIRT1 activity may be a strategy for the development of anti-HCC therapeutics.

Materials and Methods

Plasmids and antibodies

Lentivirus plasmid vectors pLKO.1-puro, and pLKO.1-puro vectors containing MISSION short hairpin RNA (shRNA) targeting SIRT1 (clone SH2421) or nontargeting shRNAs (clone SHC001) were from Sigma-Aldrich. Vectors expressing TERT (pLV102-TERT), PTOP (pLV102-TPP1), and GFP (pLV102-GFP) were from GeneCopoeia. SIRT1 (1104-1) and TERT (1531-1) antibodies were from Epitomics; POT1 (ab21382) and PTOP (ab57595) antibodies were from Abcam; p53 (#2524), acetyl-p53 (K382; #2525), phospho-H2AX (Ser139; #2577), FOXO1 (#2880), and PARP (#9542) antibodies were from Cell Signaling Technology; p16 (C-20; sc-468) and Ac-p53 (K382; sc-2525), phospho-H2AX (Ser139; sc-2577), FOXO1 (sc-2880), and PARP (sc-9542) antibodies were from Cell Signaling Technology; p16 (C-20; sc-468) and Akt (sc-49437) antibodies were from Cell Signaling Technology; p27 (54428) antibody was from BD Biosciences. p-LC3, p-PARK, p-FOXO1 (C20), and p-p53 (C-14) antibodies were purchased from Cell Signaling Technology

HCC specimens

Tumorous liver tissues and the corresponding adjacent nontumoral liver tissues were collected from 40 patients who underwent curative surgery for HCC at The Prince of Wales Hospital, Hong Kong. For these patients, the surgery was conducted in the morning after 8 hours of fasting. The patients were not subjected to any form of chemotherapy prior to the surgery. Informed consent was obtained from each patient recruited, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong. Total RNAs and proteins were extracted from these specimens.

Tissue microarrays and immunohistochemistry

The HCC tissue microarrays were generated from formalin-fixed, paraffin-embedded archival tissues of 150 paired HCC. The samples were collected at The Prince of Wales Hospital from 1995 to 2002. Tissue slides were deparaffinized, followed by quenching of endogenous peroxidase activity by hydrogen peroxide. SIRT1 antibody was applied at a dilution of 1:400. Chromogen development was done by the universal horseradish peroxidase multilink ultraview kit (Ventana Medical System). The scoring of SIRT1 was carried out by 2 independent pathologists according to the proportion of tumor cells with positive nuclear staining (negative, none; weak, <10%; moderate, 10-50%; strong, >50%).

Cell culture

HepG2, SK-Hep-1, PLC5, Hep3B, SNU-449, and SNU-423 cells were obtained from American Type Culture Collection recommendations. Huh7 cell line was acquired from the Health Science Research Resources Bank (Osaka, Japan). HKCI-4 and HKCI-2 cells were established in Prof. Nathalie Wong’s laboratory (14). Huh7, HepG2, Hep3B, and PLC5 were cultured according to recommendations in Dulbecco’s modified Eagle’s medium containing 10% FBS (Gibco BRL). SNU-449 and SNU-423 were maintained in complete RPMI medium (Gibco BRL). HKCI-4 and HKCI-2 cell lines were maintained in RPMI 1640 glutamax with HEPEs supplemented with 10% FBS, 10 μg/mL selenium, 10 μg/mL transferrin, and 10 μg/mL insulin. All cells were authenticated by short tandem repeat profiling analysis.

Lentivirus production

Lentivirus expressing shSIRT1-1, shSIRT1-2, or shCont was produced in HEK-293FT cells by using the corresponding pLKO.1-puro vector with the aid of packaging plasmids pLP1, pLP2, and pLP/VSVG from BLOCK-it Lentiviral RNAi Expression System (Invitrogen). The viruses were concentrated by using PEG-it virus precipitation solution (System Biosciences) and stored at −80°C.

RNA extraction and real-time quantitative PCR

Total RNA preparation and cDNA synthesis were carried out as described (15). Quantitative PCR (qPCR) experiments were carried out by using the SYBR Green PCR core reagent kit (Applied Biosystems). Detailed information of the qPCR reaction and the primer used are listed in the Supplementary Table S1.

Western blotting analysis

Protein lysates were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with antibodies as indicated. Blots were developed with ECL Western blotting reagents. Band intensities were quantified by ImageJ (NIH).

Cell proliferation and bromodeoxyuridine assay

Cell proliferation in response to SIRT1-silencing was determined by trypan blue exclusion assay. DNA synthesis was determined by the bromodeoxyuridine (BrdU) assay according to manufacturer’s instructions (Roche Diagnostics). The result was expressed as a percentage of the...
maximum absorbance at 450 nm, based on 3 independent experiments. The effect of doxorubicin treatment on cell proliferation was measured by the MTT assay as described previously (16).

**Colonies formation assay and soft agar assay**

The colony formation assay was carried out as described (17). Crystal violet–stained colonies were scored and results from duplicate assays were expressed as the mean from 4 independent experiments. For the soft agar assay, the base layer of each well consisted of 1.5 mL solidified media containing DEME with 10% FBS and 0.5% low melting point agarose. The top agar layer consisted of 5,000 SK-Hep-1 cells suspended in DEME with 0.35% low melting point agarose. Cells were allowed to grow for 3 weeks and the number of total colonies was counted under a microscope. Each assay was done in triplicate.

**Analysis of cell-cycle distribution and apoptosis**

Cell-cycle distribution and apoptosis were determined by fluorescence-activated cell sorting (FACS) analysis as described (18). Flow cytometry was carried out by a FACScalibur flow cytometer (BD Bioscience). Data acquisition and analysis were done with CellQuest (BD Bioscience).

**Senescence-associated β-galactosidase staining**

Senescence-associated β-galactosidase (SA-β-gal) staining of cells was done according to the method of Dimitri and colleagues (19).

**Quantification of telomere dysfunction–induced foci**

Telomere dysfunction–induced foci (TIF) was monitored by TRF2-γH2AX colocalization by using immunofluorescence (IF). Cells were fixed with 4% paraformaldehyde in PBS for 15 minutes, ice-cold methanol/acetic acid (1:1) for 30 minutes, and methanol for another 30 minutes. Cells were then permeabilized by PBST (PBS, 0.2% Triton X-100) and blocked with 10% normal goat serum. IF was carried out by using the rabbit monoclonal anti-γH2AX (ser-139) antibody (1:50) and mouse anti-TRF2 antibody (1:100) overnight at 4°C. The cells were then rinsed with PBS and incubated with anti-mouse fluorescein isothiocyanate (FITC) and goat anti-rabbit tetramethyl rhodamine isothiocyanate (TRITC) antibodies. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Digital images were captured with a Zeiss Axiovert 200 M fluorescence microscope with a Cool MPS2 (Photometrics) camera.

**Statistical analysis**

SIRT1 expression in HCC and nontumoral liver tissues were compared by the paired Student’s t test. Correlations between SIRT1 and individual clinicopathologic parameters were evaluated by the nonparametric χ² test and Spearman’s ρ rank test. The Kaplan–Meier method was used to estimate the survival rates for SIRT1 expression. The equivalences of the survival curves were tested by log-rank statistics. All statistical analyses were carried out by the statistical program SPSS version 16.0.

**Results**

**SIRT1 expression in HCC**

We first determined the expression of SIRT1 in a panel of 9 HCC cell lines and 3 normal liver biopsies. Although the level of SIRT1 was almost undetectable in the normal livers, it was significantly overexpressed in all the 9 HCC cell lines examined (Fig. 1A). To establish the relevance of SIRT1 expression in HCC, we further examined its protein expression in primary HCC and in adjacent nontumoral liver, which is often considered the premalignant lesion of HCC. Western blotting analysis indicated that a significant portion of patients showed elevated SIRT1 level in tumor tissues (28 of 40 cases; Fig. 1B). Furthermore, the average level of SIRT1 was found to be significantly higher in the tumor (median 0.25, quartiles 0.95–0.08) relative to the nontumoral liver (median 0.05, quartiles 0.07–0.03; paired t test P < 0.001; Fig. 1C). On the contrary, the analysis of SIRT1 mRNA level from these patients by real-time qPCR revealed that the average SIRT1 mRNA levels in the tumor and nontumoral liver did not differ significantly (Fig. 1D), suggesting that tumor-specific overexpression of SIRT1 was regulated in a transcription-independent manner.

Next, large-scale immunohistochemical analysis of SIRT1 expression was carried out on tissue microarrays that contained 150 early resectable HCC and paired adjacent nontumoral livers. The clinicopathologic parameters of these HCC cases are summarized in Supplementary Table S2. Distinct SIRT1 staining was found in certain tumors (46 of 150 cases), whereas the staining was negative in all adjacent nontumoral livers. In SIRT1-positive HCC, immunostaining could be identified in the tumor hepatocytes, and the staining was predominantly localized in the nucleus (Fig. 1E). SIRT1-positive tumors were further classified into weak, moderate, and strong categories according to the scores obtained by determining the percentage of positively stained cells on the section. Among the 46 positive cases, 21 showed strong staining of SIRT1, whereas 8 and 17 cases exhibited moderate and weak staining, respectively. SIRT1 expression in immunohistochemistry correlated positively with the tumor grade and weak staining, respectively. SIRT1 expression was regulated in a transcription-independent manner.

**Effect of SIRT1 silencing on HCC cell proliferation, senescence, and apoptosis**

To further elucidate the functional role of SIRT1 in HCC, we transduced HCC cells SK-Hep-1 (p53 wild-type), HepG2 (p53 wild-type), Hep3B (p53 deleted), and PLC5 (p53 inactivated) with lentiviruses containing shRNAs. Two independent shRNAs (shSIRT1-1 and shSIRT1-2) showed efficient SIRT1 knockdown in these cell lines (>90%) compared with the untreated or scramble shRNA (shCont)-transduced cells (Fig. 2A).

Downregulation of SIRT1 resulted in a marked decrease in cell number of all cells tested over a course of 6 days, independent of their p53 status (Fig. 2B; Supplementary...
Furthermore, knockdown of SIRT1 reduced the number and size of SK-Hep-1 cell colonies as determined by colony formation assays (Fig. 2C). It also inhibited anchorage-independent growth of the cells as determined by soft agar assays (Fig. 2D). Concordantly, SIRT1 knockdown reduced DNA synthesis as measured by BrdU incorporation (Fig. 2E). Cell-cycle analysis showed that significant G1 arrest was observed in SK-Hep-1 and HepG2 cells, whereas G2 arrest was observed in PLC5 and Hep3B cells (Fig. 2F). Taken together, these data suggest that while reduced levels of SIRT1

**Figure 1.** Expression of SIRT1 in HCC. A, expression of SIRT1 protein was analyzed by Western blot analysis by using SIRT1 antibodies. β-Actin was used as a loading control. B, level of SIRT1 in 40 paired HCC by Western blot analysis by using SIRT1 antibodies. β-Actin was used as a loading control. A and B, total protein extracts were used for Western blotting analysis. C, quantification of SIRT1 protein expression in HCC and their adjacent nontumoral livers. *, P < 0.001. D, real-time qPCR analysis of SIRT1 mRNA expression in HCC and their adjacent nontumoral livers. β-Actin mRNA expression was used as an internal control. E, immunohistochemical examination of SIRT1 expression in primary and paired adjacent nontumoral HCC. Magnification, ×400. Inset: magnification, ×100.
repressed cell proliferation in general, it has disparate effects on cell-cycle distribution in different HCC cells.

An earlier study showed that inhibition of SIRT1 leads to senescence-like growth arrest in breast and lung cancer cells (20). We also observed that gene silencing of SIRT1 in SK-Hep-1 and HepG2 (p53 wild-type) resulted in cells that were enlarged in size, flattened in shape, and highly positive for SA-β-gal staining, whereas negative staining was observed in Hep3B and PLC5 (p53 null or mutated) cells (Fig. 3A). SIRT1 silencing was associated with increased acetylation of FOXO1 (Supplementary Fig. S2), a well-known SIRT1 substrate, suggesting that there is a concordant reduction of SIRT1 expression and activity. Cellular senescence in SK-Hep-1 and HepG2 cells was associated with enhanced p53 acetylation on lysine 382 and induction of p21 that are known for their role in senescence induction (21). Enhanced p53 acetylation, but not p21 induction, was observed in PLC5 cells, consistent with its mutated p53 status. On the contrary, the expression of 2 other proteins implicated in senescence, namely p27 and p16, was not changed significantly on SIRT1 knockdown (Fig. 3B).

Increased propensity to apoptotic cell death was evidenced by the enhanced PARP cleavage in Hep3B and PLC5 cells, compared with HepG2 and SK-Hep-1 cells (Fig. 3C). Collectively, these data suggest that SIRT1 knockdown is associated with G1 arrest and cell senescence in p53 wild-type HCC cells, but leads to G2 arrest and apoptosis in p53 mutated HCC cells.

### Table 1. Correlation of SIRT1 expressions with clinicopathologic features

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NOTE: Strong SIRT1 protein score was associated with progressive advancement of histologic differentiation.
The role of SIRT1 in telomeric maintenance

Telomeres are nucleoprotein structures that protect the ends of chromosomes. Mammalian telomeres are protected from being recognized as sites of DNA damage by the shelterin complex, which is composed of TRF1, TRF2, POT1, TIN2, PTP, and RAP1 (22). Either critically shortened telomeres or the inhibition of shelterin causes telomeric dysfunction that is characterized by the activation of DNA damage response.
and the formation of TIF, leading to cellular senescence (23, 24). To determine whether the observed cellular senescence or cell death is associated with telomeric dysfunction, we evaluated the expression of telomerase (TERT), members of the shelterin complex, and other telomere-associated proteins (PINX1, TANK1, and KU-70) by real-time qPCR.

Compared with untreated or shCont-expressing cells, SK-Hep-1 cells expressing SIRT1 shRNAs resulted in a significant reduction of TERT, POT1, and PTOP mRNA (Fig. 4A). Concordantly, a marked reduction of TERT and PTOP protein expression on SIRT1 knockdown was observed in all hepatoma cells examined (SK-Hep-1, HepG2, Hep3B, and PLC5), suggesting that SIRT1 may play an important role in PTOP and TERT expression in liver tumors (Fig. 4B). A reduction of POT1 expression on SIRT1 knockdown was observed only in SK-Hep-1 and Hep3B cells but not in HepG2 and PLC-5 cells, suggesting that the POT1 gene may be subjected to differential regulation in different cells (Fig. 4C).

Importantly, when PTOP or TERT, but not GFP, was ectopically expressed in cells transduced with lentiviruses expressing shSIRT1-1 or shSIRT1-2 (Fig. 4C), a significant enhancement in cell growth (Fig. 4D) and a reduction in cell senescence (Supplementary Fig. S3) were observed in SK-Hep-1 cells. Similarly, ectopic expression of PTOP or TERT also promoted cell growth in SIRT1-depleted Hep3B cells (Supplementary Fig. S4). Together, these data supported the notion that PTOP and TERT depletion are responsible, at least in part, for cell growth suppression by SIRT1 knockdown in HCC cells.

PTOP plays an essential organizing function in shelterin and protects telomeres from TIF via recruiting POT1 (25). It also recruits TERT to the telomere and regulates its activity.
Figure 4. SIRT1 knockdown induces telomeric dysfunction. A, real-time qPCR analysis of gene expressions related to telomeric maintenance. SK-Hep-1 cells were transduced with lentiviruses expressing the indicated shRNA. β-Actin mRNA expression was used as an internal control. Experiment was conducted in triplicate. Values represent the mean ± SD of 3 independent experiments. *, P < 0.001. B, Western blot analysis of SIRT1, TERT, PTOP, POT1, and β-actin expressions of SK-Hep-1 cells. Cells were transduced with lentiviruses expressing the indicated shRNA for 5 days before analysis. C–E, effects of TERT or PTOP overexpression on cells depleted for SIRT1. SK-Hep-1 cells were transfected with TERT-, PTOP-, or GFP-expressing vector. Cells were grown for 1 week under the selection of puromycin (0.25 μg/mL). Subsequently these cells were transduced with lentivirus expressing shCont, shSIRT1-1, and shSIRT1-2. Cells were harvested for Western blot analysis (C) or subjected to cell proliferation assay (D) after 6 days. D, values were expressed relative to the cell count in the shCont group, which was set to 100%. Bars represent the mean ± SD of 3 independent experiments. E, left, induction of TIF by knockdown of SIRT1. SK-Hep-1 cells at 4 days after lentiviral transduction were analyzed by using TRF2 antibodies for telomeres (TRITC, red) and γ-H2AX antibodies for DNA damage (FITC, green). Arrow indicates telomeric γ-H2AX foci. Right, quantification of the TIF response. Cells shown were scored for 5 or more telomeric γ-H2AX foci. Bars represent the mean ± SD of 3 independent experiments. F, left, SK-Hep-1 cells at 4 days after lentiviral transduction were analyzed by using α-tubulin antibodies (TRITC, red) and DAPI (blue). Arrow denotes multinuclei; arrow head denotes micronuclei. Right, quantification of nuclear abnormalities. 100 cells were randomly picked from 5 fields and scored. Bars represent the mean ± SD of 3 independent experiments.
Gene knockdown of PTOP results in the activation of DNA damage signaling pathway, leading to telomeric fusion detectable in metaphase spreads (25). Consistent with these known functions of PTOP, we observed that SIRT1 silencing significantly induced the formation of TIF in SK-Hep-1 cells as evidenced by colocalization of phosphorylated H2AX (γ-H2AX) and TRF2 (Fig. 4E). However, we could not analyze telomeric fusion in cells (SK-Hep-1, PLC5, HepG2, and Hep3B) expressing SIRT1 shRNA, because these cells failed to undergo mitotic arrest in response to colchicine treatment (data not shown). We further showed that SIRT1 depletion probably disrupted mitotic checkpoint signaling because either one or more genes involved in mitotic arrest, including AURK-A, AURK-B, CENP-A, and BUB1, were repressed on SIRT1 knockdown (Supplementary Fig. S5). In line with this observation, cells expressing SIRT1 shRNA exhibited increased nuclear abnormality characterized by multinuclei and micronuclei formation (Fig. 4F). Collectively, these data suggested that telomeric dysfunction and genetic instability are the major contributing factors to cell growth suppression induced by SIRT1 reduction.

Correlation between SIRT1, TERT, and PTOP expression in human HCC

To determine the relevance of the earlier described SIRT1-regulated pathways in human subjects, we probed for the expression of TERT and PTOP in HCC by Western blotting by using the same 40 paired HCC that have been used (Fig. 1B) to determine SIRT1 expression. Overall, TERT was expressed at a higher level in HCC compared with adjacent nontumoral liver tissues, whereas PTOP was expressed at a comparable level in these tissues (Fig. 5A). Correlative analysis further revealed a significant association between tumoral induction of TERT and SIRT1 (Spearman’s rank = 0.40, P = 0.01; Fig. 5B), whereas the association between tumoral induction of SIRT1 and PTOP was not apparent (Spearman’s rank = 0.08, P = 0.21; Fig. 5C). These data suggest that a SIRT1-TERT regulatory axis may exist in vivo.

SIRT1 knockdown enhanced cytotoxicity of doxorubicin in HCC cells

To further show the potential of targeting SIRT1 for HCC therapy, we determined whether SIRT1 inhibition enhances the antitumor effect of the DNA-damaging agent doxorubicin, the only clinically efficacious chemotherapeutic agent for HCC treatment. Cells were first transduced with lentiviruses expressing shSIRT1-1 and shSIRT1-2 for 2 days, before doxorubicin was added to the medium at a final concentration of 0 to 12 μmol/L and incubated for 48 hours. Remarkably, reduced SIRT1 expression increased the chemosensitivity of SK-Hep-1 and PLC5 cells to doxorubicin treatment by 8- and 4-fold, respectively (Fig. 6A). Concor- 
dantly, reduced SIRT1 expression also promoted doxorubicin-induced apoptosis of these cells, as evidenced by enhanced PARP cleavage (Fig. 6B). Together, these data suggest the therapeutic potential of combining a SIRT1 inhibitor and doxorubicin in the treatment of a subset of HCC in which SIRT1 is upregulated.

Discussion

The role of SIRT1 in tumorigenesis is controversial. Increased SIRT1 expression has been found to reduce tumor formation in a mouse model of colon tumor (28), whereas SIRT1 mutant mice exhibited increased DNA instability and are more susceptible to tumor development (29). Reduced levels of SIRT1 mRNA and proteins are observed in breast tumors compared with normal tissue (29). Intriguingly, however, SIRT1 overexpression is found in acute myeloid leukemia (30), prostate (31), skin (32), gastric (33), and colorectal cancers (34), suggesting a tumorigenic role. Therefore, the function of SIRT1 may be tumor-type specific and may also depend on the stage of tumorigenesis being assessed.

With regard to HCC, Wang and colleagues (29) found that SIRT1 mRNA is expressed at a comparable level in both tumor and nontumoral tissues, by analyzing pooled microarray data from HCC samples. They further concluded that SIRT1 protein expression is reduced in HCC on the basis of the analysis of one paired HCC specimen (29). In agreement with their results, our study confirmed that SIRT1 mRNA levels do not differ between HCC and nontumoral tissues. However, by analyzing SIRT1 protein expression with our large collection of paired frozen HCC tissues and histologic sections, we have convincingly shown that SIRT1 protein was indeed overexpressed in a subset of HCC by a posttranscriptional mechanism. We have also shown that SIRT1 expression is low in normal and premalignant livers, and its positivity is closely associated with poorly differentiated histology. Furthermore, reduced levels of SIRT1 suppressed cell proliferation and anchorage-independent growth of HCC cells. Together, our data support the notion that SIRT1 overexpression may play a role in HCC tumorigenesis.

Recent studies also suggested that inhibition of SIRT1 may have anticancer potential. RNAi-mediated silencing of SIRT1 genes resulted in growth arrest or apoptosis in some epithelial tumor cells (35), and reactivated tumor suppressor genes (36). The SIRT1 inhibitor, sirtinol, induced senescence-like growth arrest in tumor cells (20), whereas another inhibitor, camadinol, induced apoptosis of Burkitt lymphoma cells and suppressed the growth of tumor xenografts in vivo (37). Our study revealed that the knockdown of SIRT1 in HCC cells resulted in the apoptosis or the characteristic senescence-like growth arrest phenotype closely resembling that of breast and lung cells treated with sirtinol (20), suggesting that reduced SIRT1 activity may perturb pathways essential for cell proliferation in these cancer cells.

We further identified telomeric dysfunction as one of the major phenotypes of reduced SIRT1 levels in HCC tumor cells. Telomeres are shortened on every DNA replication cycle due to the requirement of short RNA to prime DNA synthesis by DNA polymerases (38). Progressive telomere shortening is also found in chronic liver injury and liver cirrhosis (39). Telomere shortening triggers the DNA damage response, leading to cell-cycle arrest, senescence, or apoptosis (40), and it also serves as a tumor suppressor mechanism to limit the proliferation of transformed cells. Nevertheless, TERT is activated in most tumors including HCC to overcome the telomere shortening process. In line with this, we and others have convincingly shown that SIRT1 protein was indeed downregulated in most tumors (26, 27). This finding is consistent with previous reports showing that SIRT1 expression is low in most tumors including HCC to overcome the telomere shortening process.
barrier by adding back telomere repeats to chromosome ends (39). Inhibition of TERT shortens telomeres and causes cancer cell death (41). TERT antagonists also inhibit tumor growth in a xenograft animal model of HCC (42).

Although TERT plays a pivotal role in tumorigenesis, and we have showed that the proliferation of SIRT1-depleted HCC cells was rescued by TERT expression, we believe that telomere phenotypes in SIRT1-depleted cells did not result from telomeric attrition. This is because telomere shortening is a gradual process that requires a substantial number of cell division cycles for its effect on cell growth to become apparent. Therefore, the acute cell growth suppression effect as a result of SIRT1 knockdown argues against telomere attrition as the major mechanism. Indeed, telomeric length did not differ significantly between the control and SIRT1-depleted cells, as measured at 7 days after lentiviral transduction (Supplementary Fig. S6). Instead, our evidence suggests that TERT may contribute to cell survival via telomere-independent mechanisms (43, 44), and therefore reduced expression of TERT in SIRT1 knockdown cells suppresses cell proliferation that can be rescued by its reexpression. Apparently, the TIF and nuclear abnormalities observed in SIRT1 knockdown cells are more likely due to the depletion of PTOP, or both PTOP and POT1. PTOP binds to and recruits POT1 to the telomere which is essential for telomere protection. The PTOP–POT1 complex also recruits TERT and is essential for its activity (26). Overexpression of telomere-binding proteins is also found in many cancers (45, 46). Presumably these proteins are required to cope with the increased levels of cell division in tumors. Consistent with this notion, the depletion of telomeric-binding proteins, including PTOP and POT1, induces the DNA-damage response, leading to cell growth inhibition (47, 48).

Figure 5. A, expression of SIRT1, TERT, PTOP, and β-actin expression in HCC and adjacent nontumoral liver tissues. B and C, SIRT1, TERT, and POTP expression level was first normalized by the expression level of β-actin, and the fold induction of each of these proteins in HCC over nontumoral liver in each patient was calculated. Fold induction of SIRT1 was plotted against TERT (C) and PTOP (D), and was analyzed by Spearman’s r rank test.
The current understanding of the prosurvival function of SIRT1 is its role to direct the cells toward DNA repair via modulating the activity of MRE11-RAD50-NBS1, p53, Ku70, FOXO proteins, and others (49). Our findings extend the current understanding of the function of SIRT1 to include telomeric maintenance in HCC cells, and that its acute depletion could suppress tumor cell growth. It should be noted that SIRT1 depletion also impinges on other cellular processes including the repression of genes involved in mitotic checkpoint signaling. The underlying mechanistic basics remain to be determined. With regard to cancer therapy, the disruption of telomeric maintenance via targeting telomerase is one of the emerging antitumor strategies. However, one major limitation of using telomerase inhibitors for tumor therapy is the long lag time for its action, during which the tumor burden may have already increased substantially (41, 42). Therefore, targeting SIRT1 may be a more efficient strategy to induce telomeric dysfunction and tumor cell death. The response rate of HCC to various chemotherapies has been low. Our work suggests that SIRT1 may serve as a potential target for the development of anti-HCC therapeutics, and inhibition of SIRT1 may be a novel strategy to target a subset of HCC patients in whom this protein is overexpressed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgment

We thank Dr. Pauline Chiu and Dr. D.Y. Jin for critical comment on the manuscript.

Grant Support

This work is supported by the Research Grant Council Grants CUHK 466108 and 467210 to B.C.B. Ko. Laboratory of A.W.I. Lo is supported by HKRGC (CUHK 4712/07M).

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Received November 29, 2010; revised March 4, 2011; accepted April 4, 2011; published OnlineFirst April 28, 2011.

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Figure 6. SIRT1 knockdown sensitizes HCC cells to doxorubicin treatment. A, SK-Hep-1 and PLC5 cells transduced with lentivirus expressing the indicated shRNA were treated with doxorubicin at various concentrations for 2 days, and then processed for MTT assay. B, SK-Hep-1 and PLC5 cells transduced with lentivirus were treated with 0.75 μmol/L of doxorubicin for 2 days. Cells were harvested for PARP cleavage analysis by using PARP antibodies. β-Actin was used as a loading control.


Correction: Sirtuin 1 Is Upregulated in a Subset of Hepatocellular Carcinomas where It Is Essential for Telomere Maintenance and Tumor Cell Growth

In this article (Cancer Res 2011;71:4138–49), which was published in the June 15, 2011, issue of Cancer Research (1), the third to last sentence in the abstract contained a typographical error. The corrected sentence is listed below. The publisher regrets this error.

There was also a positive correlation between the level of induction of SIRT1 and TERT in human HCC.

Reference


Published OnlineFirst January 25, 2012. doi: 10.1158/0008-5472.CAN-12-0033 ©2012 American Association for Cancer Research.
Sirtuin 1 Is Upregulated in a Subset of Hepatocellular Carcinomas where It Is Essential for Telomere Maintenance and Tumor Cell Growth

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Cancer Res 2011;71:4138-4149. Published OnlineFirst April 28, 2011.

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