Modulation of SIRT3 expression through CDK4/6 enhances the anti-cancer effect of sorafenib in hepatocellular carcinoma cells

HanHee Jo

Incheon National University

Dayoung Yoo

Incheon National University

JongSook Lee

Incheon National University

Seon yoo Kim

Yonsei University College of Medicine

Jee-in Chung

Yonsei University College of Medicine

Hae yong Ko

Yonsei University College of Medicine

Misu Lee (✉ misulee@inu.ac.kr)

Incheon National University <https://orcid.org/0000-0001-9208-2127>

Mijin Yun

Yonsei University College of Medicine

Research article

Keywords: Hepatocellular carcinoma, SIRT3, sorafenib, anti-tumor effect, drug sensitivity, CDK4/6 inhibitor

Posted Date: October 17th, 2019

DOI: <https://doi.org/10.21203/rs.2.16163/v1>

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Version of Record: A version of this preprint was published at BMC Cancer on April 19th, 2020. See the published version at <https://doi.org/10.1186/s12885-020-06822-4>.

Abstract

Background : Hepatocellular carcinoma (HCC) is the fifth leading cause of cancer-related deaths worldwide. The only drug currently approved for clinical use in the treatment of advanced HCC is sorafenib. However, many patients with HCC show reduced sensitivity to sorafenib during treatment. SIRT3, a member of the mammalian sirtuin family, is a tumor suppressor in certain tumor types. To date, a few studies have investigated the effects of SIRT3 on tumor prognosis and sorafenib sensitivity in patients with HCC. Thus, this study aimed to investigate the correlation between SIRT3 expression and glucose metabolism and proliferation in HCC and to discover effective compounds that increase endogenous SIRT3 modulation effect of sorafenib. **Methods:** To determine the correlation between SIRT3 and glucose related proteins, immunostaining was performed with liver cancer tissue using various antibodies. To investigate whether the expression of SIRT3 in HCC is related to the resistance to sorafenib, we treated sorafenib after the modulation of SIRT3 levels in HCC cell lines (overexpression in Huh7, knockdown in HepG2). We also employed PD0332991 to modulate the SIRT3 expression in HCC cell and conducted functional assays. **Results:** SIRT3 expression is downregulated in high glycolytic and proliferative HCC of human patients, xenograft model and HCC cell lines. We also demonstrated that SIRT3 expression was downregulated after sorafenib treatment, resulting in reduced drug sensitivity in HCC cell lines. To enhance the anti-tumor effect of sorafenib, we employed PD0332991 (CDK4/6-Rb inhibitor) based on the correlation between SIRT3 and phospho-Rb in HCC. In addition, combined treatment with sorafenib and PD0332991 showed a synergic anti-tumor effect in HCC cells. **Conclusions:** Taken together, our data suggest that the modulation of SIRT3 by CDK4/6 inhibition might be used for HCC therapy together with sorafenib, which, unfortunately, has limited efficacy and whose use is often associated with drug resistance.

Background

HCC is one of the leading causes of cancer-related deaths worldwide (1). Patients with early-stage HCC are asymptomatic; hence, HCC is usually detected at intermediate or advanced stages, in which patients cannot receive curative treatments such as ablation, surgical resection, or liver transplantation (LT) (2). Although surgical treatment has improved the disease outcome, the risk of recurrence remains substantial even for early HCC. In patients with advanced HCC, sorafenib (Nexavar), an orally active multikinase inhibitor, has been used as a first-line chemotherapeutic agent (3). Despite extending the median survival by 3–5 months, the high resistance rate and serious adverse side effects have significantly limited the benefits of sorafenib therapy (4-6). Therefore, there is an increasing need for a strategy to enhance the effects of sorafenib anti-cancer activity.

Sirtuins (SIRT1-7) have emerged as important regulators of tumorigenic processes such as proliferation, cell cycle progression, cell survival, metabolism, and angiogenesis (7-9). SIRT3, the best characterized mitochondrial sirtuin, deacetylates and activates several enzymes involved in cellular redox balance and defense against oxidative damage (10-12). Several reports suggest that SIRT3 has a dual role in cancer (13-15). SIRT3 functions as an oncogene in oral cancer and melanoma by keeping ROS levels under a

certain threshold to prevent apoptosis and promote cell proliferation (16, 17). In contrast, SIRT3 has been identified as a tumor suppressor in hepatocellular carcinoma (HCC)(18, 19), breast cancer (20), ovarian cancer (21), and leukemia (22). Further, it has been reported that SIRT3 plays a role in metabolic reprogramming (Warburg) and in triggering cell death under stress conditions (23, 24). Indeed, high SIRT3 expression is correlated with favorable outcomes and an increase in the overall survival rate of patients with HCC (25). In this regard, regulation of SIRT3 expression might be a novel strategy to investigate more personalized therapies against cancers. In addition, SIRT3 expression levels affect sensitivity to chemotherapeutic agents in HCC (26).

In this study, we purposed to investigate the correlation between SIRT3 expression and glucose metabolism and proliferation in HCC. In addition, because a few compounds have been explored to modulate SIRT3 activity (27, 28), we also aimed to discover effective compounds that increase endogenous SIRT3 modulation of the anti-cancer effect of sorafenib.

Methods

Human HCC samples

This study was approved by the Institutional Review Board at Yonsei University Health System Severance Hospital (Seoul, South Korea), and the study was conducted using the current guidelines for ethical research (Yonsei IRB number: 4-2015-0904). The selection of patients was performed as described previously (29).

Chemicals

PD0332991 was purchased from TOCRIS Bioscience (Bristol, UK) and sorafenib was purchased from Santa Cruz (Dallas, TX, USA). PD0332991 and sorafenib were dissolved in DMSO (Sigma Aldrich, St. Louis, MO, USA) at a concentration of 10 mM. All reagents were stored at -80 °C.

Cell lines and cell culture

The human HCC cell lines HepG2, Hep3B, skHep1, and Huh7 were purchased from the Korean Cell Line Bank. HepG2 was cultured in RPMI, and Hep3B, skHep1, and Huh7 were cultured in DMEM. All media were supplemented with 10% FBS (Hyclone) and 1% penicillin streptomycin. Cells were maintained in a humidified incubator with 5% CO₂ at 37 °C. Hep3B, skHep1, and Huh7 cell lines were plated and incubated for 24 h before transfection. Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) was used to perform siRNA transfection following the manufacturer's instructions.

Cell proliferation assay and glucose measurement

WST-1 colorimetric assays (Roche, Mannheim, Germany) for cell viability were performed 48 h after treatment according to the manufacturer's recommendations. Huh7 cells were placed in 96-well plates and transfected with MOCK or pcDNA-SIRT3 plasmid. After 48 h of treatment, we measured glucose uptake using Glucose Assay (Promega, Germany) according to the manufacturer's recommendation. Absorbances at 440 nm and 640 nm were measured using a microplate reader (Molecular Devices, CA, USA).

RNA isolation and sequencing

Total RNA was isolated using TRIzol reagent (Invitrogen). RNA quality was assessed by Agilent 2100 bioanalyzer using the RNA 6000 Nano Chip (Agilent Technologies, Amstelveen, The Netherlands), and RNA quantification was performed using ND-2000 Spectrophotometer (Thermo Inc., DE, USA). For control and test RNA samples, library was constructed using QuantSeq 3' mRNA-Seq Library Prep Kit (Lexogen, Inc., Austria) according to the manufacturer's instructions. In brief, 500 ng total RNA was prepared for each sample, an oligo-dT primer containing an Illumina-compatible sequence at its 5' end was hybridized to the RNA, and reverse transcription was performed. After degradation of the RNA template, second strand synthesis was initiated by a random primer containing an Illumina-compatible linker sequence at its 5' end. The double-stranded library was purified using magnetic beads to remove all reaction components. The library was amplified to add the complete adapter sequences required for cluster generation. The amplified library was purified, and high-throughput sequencing was performed as single-end 75 sequencing using NextSeq 500 (Illumina, Inc., USA).

Real-time PCR

Total RNAs were extracted with TRIzol (Invitrogen). cDNA was synthesized from 500 ng of total RNA using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Quantitative RT-PCR was conducted on C1000 a Thermal Cycler (Bio-Rad) using SYBG Green Real-time PCR Master Mix (Toyobo, Osaka, Japan). Gene expression levels were normalized with beta-2 microglobulin (B2M) mRNA expression levels of corresponding cDNA samples. All PCR primers were purchased from Bioneer (Daejeon, Korea). The following primers were used: SIRT3 (Forward 5'-GAAACTACAAGCCCAACGTCA-3', Reverse 5'-AAGGTTCCATGAGCTTCAACC-3'), RB1 (Forward 5'-GAAGCAACCCTCCTAAACCAC-3', Reverse 5'-CTGCTTTTGCATTTCGTGTTTCG-3'), and B2M (Forward 5'-TTACTCACGTCATCCAGCAGA-3', Reverse 5'-AGAAAGACCAGTCCTTGCTGA-3').

Western blotting

Western blotting was performed as described previously (29). The primary antibodies in the present study were: SIRT3 (Cell Signaling Technology, Danvers MA, USA; clone C73E3; dilution 1:1000), CDK4 (DCS156, 1:1000), CDK6 (DCS83, 1:1000), Phospho-Rb (Ser807/811) (D20B12, 1:1000), Rb (4H1, 1:2000), PCNA (D3H8P, 1:2000), GLUT1 (1:2000) from Abcam (Cambridge, UK), and Ki67 (Santa Cruz, Dallas TX, USA; MIB-1, 1:500). Western blotting experiments from biological replicates showed similar expression data, attesting to the reproducibility of the results. For band quantification, images were analyzed using Image Lab software (Bio-Rad, Hercules, California, USA).

Immunostaining

Immunohistochemistry (IHC) and immunofluorescence (IF) were performed as described previously (30). After antigen retrieval, immunostaining was performed using various antibodies. The primary antibodies used were: SIRT3 (Cell Signaling Technology, Danvers MA, USA; clone C73E3; dilution 1:500); Ki67 (Dako, Glostrup, Denmark; MIB-1; 1:500); GLUT1 (1:500), and Ki67 (SP6, 1:500) from Abcam (Cambridge, UK). Images were recorded using an Olympus BX53 microscope with Olympus Cell Sens software (Carl Zeiss Microscopy, GmbH, Jena, Germany).

The Cancer Genome Atlas (TCGA) data analysis

mRNA levels of TCGA liver HCC data were obtained from the OncoLnc TCGA data portal (www.oncolnc.org). A set of 360 HCC samples with high and low gene expression groups (50-50 percentile) was used for correlation graphs of two different genes. GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) was used for mapping.

Statistical analysis

A paired two-tailed Student's t-test was used to detect significant differences between the two sets of data, and $p < 0.05$ was considered statistically significant.

Results

Expression of SIRT3 in patients with HCC

Imaging with 18F-fluorodeoxyglucose (FDG) positron emission tomography/computed tomography (PET/CT) was used to evaluate glucose metabolism. To investigate the correlation between glucose uptake and SIRT3 expression, twenty-one patients with HCC were divided into two groups according to 18F-FDG uptake: 9 patients with high glycolytic HCC with high 18F-FDG uptake and 12 with low glycolytic HCC with low 18F-FDG uptake. The mRNA expression of *SIRT3* was higher in the low glycolytic group

than in the high glycolytic group (Fig 1A). To confirm our observation, we performed immunohistochemistry (IHC) analysis with HCC tissues in two groups. In patients in the low FDG uptake group, low GLUT1 expression, low Ki67 expression, and high SIRT3 expression were observed in the tumor region. High Ki67 and low SIRT3 expression levels were observed in patients with high GLUT1 expression in the high FDG uptake group (Fig 1B). Thus, SIRT3 expression seemed to be associated with glycolytic metabolism and also cell proliferation in patients with HCC.

Differential expression of SIRT3 in HCC cells

The expression of SIRT3 was assessed in four HCC cell lines and their xenograft models with different rates of proliferation and glycolysis. As shown in Fig 2A and 2B, HepG2 cells had the highest expression of SIRT3 at the mRNA and protein levels. Similar to human patients with HCC, an inverse correlation between GLUT1 and SIRT3 was observed in an HCC xenograft model (Fig 2C). Moreover, there was a significant negative correlation between SIRT3 and Ki67. HepG2 and Hep3B cells had low Ki67 and high SIRT3 expression, whereas Huh7 and SK-HEP1 cells had high Ki67 and low SIRT3 expression. The results were consistent with the TCGA database analysis of human HCC samples (Supporting data 1A and 1B). Spearman's correlation also showed a significant negative correlation between *SIRT3* and *Ki67* (Spearman's coefficient $r = -0.3093$, $P < 0.0001$), and *SIRT3* and *HK2* (Spearman $r = -0.239$, $P < 0.0001$). To further evaluate our findings, SIRT3 was overexpressed in Huh 7 cells with low basal SIRT3 expression. Overexpression of SIRT3 reduced the expression of Ki67 and GLUT1 and also significantly reduced glucose uptake (Fig 2D and 2E). Thus, we confirmed the negative correlation between SIRT3 expression and glycolytic metabolism and proliferation in HCC cells and in their xenograft model.

Reduction of SIRT3 expression after sorafenib treatment

Because we determined a crucial role of SIRT3 in HCC, we wanted to investigate whether the expression of SIRT3 was changed by sorafenib treatment. For this purpose, HCC cell lines were incubated with sorafenib for 48 h. We observed that the expression of SIRT3 in HCC cells decreased after treatment with 10 μ M sorafenib (Fig 3A). Furthermore, a more prominent decrease in cell proliferation was observed in cells transfected with SIRT3 than in cells transfected with MOCK (Fig 3B and 3C) in Huh7 cells, suggesting that the expression of SIRT3 enhances the sensitivity of HCC cells to sorafenib. Modulation of SIRT3 might be an effective strategy to increase the sensitivity to sorafenib.

Correlation between SIRT3 and phospho-Rb (pRb)

PD0332991, a highly selective inhibitor of CDK4/CDK6 kinases with the ability to block phosphorylation activity of Rb, has become a novel therapeutic candidate for HCC (31). We assessed the expression of

pRb in patients with HCC to determine whether PD0332991 can be a candidate drug to increase the sensitivity of sorafenib by modulation of SIRT3 expression. We performed IHC using antibody specific for pRb using HCC xenograft model. Huh7 and skHep1-xenograft models with low SIRT3 expression showed high expression of pRb. Thus, the expression of pRb and SIRT3 had a negative correlation in HCC xenograft models (Fig 4A). In human HCC, Rb mRNA levels were significantly higher and those of SIRT3 mRNA relatively lower, in the high 18F-FDG group than in the low 18F-FDG group (Fig 4B). Spearman's correlation also showed a significant negative correlation between *SIRT3* and *Rb1* (Spearman's coefficient $r = -0.3408$, $P < 0.0001$) expression levels in human HCC patients by TCGA data analysis (Supplementary Fig 1D). Moreover, we also observed a negative correlation between the expression of pRb and SIRT3 in human patients with HCC (Fig 4C).

SIRT3 expression is upregulated upon treatment with a CDK4/6 inhibitor

Because a connection between SIRT3 and pRb was identified in this study, we investigated whether PD0332991 can modulate the expression of SIRT3 to enhance the sorafenib sensitivity in HCC cells. Upon PD0332991 treatment, the expression of SIRT3 increased in HepG2. In SK-HEP1 and Huh 7 cells, SIRT3 expression increased marginally compared to that in the control (Fig 5A). However, no significant change was observed in Hep3B cells. It is possible that Hep3B cells present mutations of Rb that underlie the relative resistance to CDK4/6 inhibition (32, 33). Therefore, Hep3B cells were excluded from further experiments. Among the three HCC cells, HepG2 cells showed the highest reduction in cell proliferation after PD033291 treatment compared with SK-Hep1 and Huh7 cells (Supporting data 2). In addition to PD0332991 treatment, we investigated the expression of SIRT3 upon knockdown of *CDK4/6* in HCC cells. Similar to that in PD0332991 treatment, SIRT3 expression was upregulated in CDK4/6 knockdown HepG2 cells (Fig 5B). The expression of PCNA, a proliferation marker, decreased upon *CDK4/6* silencing, which had an effect similar to that of treatment with PD0332991 (Fig 5B).

Our data suggested that SIRT3 expression is negatively correlated with glucose metabolism (Fig 1A and 1B). Thus, we assessed the expression of glycolysis- and TCA-related genes after CDK4/6 inhibition in HepG2 cells by microarray analysis. A reduction in the expression of glycolysis-related genes, including *SLC2A1* (fold change: 0.12), *PFKP* (fold change: 0.341), *PKM* (fold change: 0.457), and *HK2* (fold change: 0.693) was observed in CDK4/6 KD HepG2 cells (Fig 5C). In addition, the most dysregulated genes in the two sample groups (scramble vs. *CDK4/6* KD) were associated with the following categories: DNA replication, meiotic cell cycle process, chromosome segregation, regulation of fatty acid oxidation, lipid catabolic process, and regulation of lipid catabolic process (Supporting data 3). The rate of dysregulation in glycolysis-related genes after PD0332991 treatment was smaller compared with that after CDK4/6 KD (Fig 5C). Thus, we investigated a novel mechanism to modulate SIRT3 expression by CDK4/6 inhibition, resulting in the inhibition of glycolysis and cell proliferation.

We aimed to investigate whether upregulation of SIRT3 by the CDK4/6 inhibitor PD0332991 could enhance the anti-cancer effect of sorafenib on HCC cells. We performed combination treatment with sorafenib and PD0332991 in HepG2 and Huh7 cells. Both *SIRT3* mRNA and SIRT3 protein expression were upregulated in HepG2 cells exposed to the two drugs (Fig 6A and 6B). In these conditions, we also noticed a more pronounced reduction of cell viability compared with single treatment (Fig 6C). Huh7 cells showed the same results as HepG2 with the exception of SIRT3 mRNA expression because of the very low basal level of SIRT3 mRNA in Huh7 cells (Fig 6D and 6E). Altogether, these results show that upregulation of SIRT3 by CDK4/6 inhibition enhances the sensitivity to sorafenib treatment in HCC cells.

Discussion

Several studies have emphasized the importance of SIRT3 in carcinogenesis (9, 24, 34). However, there have been few studies on the mechanisms that control SIRT3 expression or on the discovery of clinically applicable drugs that can modulate it. In this study, we investigated a novel function of CDK4 /6 inhibitor as an inducer of SIRT3, resulting in enhanced sensitivity to sorafenib treatment in HCC cells.

To date, *SIRT3* is known as a tumor suppressor gene (23), and overexpression of SIRT3 reduces cell growth and proliferation in HCC (23, 25, 26). SIRT3 also induces apoptosis in abnormal cells through the upregulation of MnSOD, p53, Bax, and Fas (19). Wang *et al.* determined patient survival and outcome in patients with HCC according to SIRT3 expression (35). In fact, reduced expression of SIRT3 was associated with poor prognosis, whereas intra-tumoral SIRT3 expression is a good prognostic factor in the early stages. So far, the effect of SIRT3 on glucose metabolism has been studied in cancers other than HCC (36-38). Finley *et al.* have proposed that SIRT3 loss increases ROS levels and promotes tumorigenesis by altering global cellular metabolism (24). In this study, patients with HCC were divided into low and high glycolytic groups by 18F-FDG-PET analysis. Consistent with previous results with other tumor types, we found high SIRT3 and low Ki67 expression in the low glycolytic group of patients with HCC. Thus, our study suggests that SIRT3 expression is associated with glycolysis and proliferation in human HCCs. Indeed, upregulation of SIRT3 by CDK4/6 inhibition and treatment with PD0332991 induced the downregulation of glycolysis-related genes in our gene analysis.

Selective CDK4/6 inhibitors, including PD0332991, are currently used for the treatment of a variety of tumor types, including breast cancer, melanoma, and non-small cell lung cancer (39-41). In this study, we found that CDK4/6 inhibition by treatment with siCDK4/6 or PD0332991 upregulated SIRT3 expression. Previous studies have determined that SIRT1 is involved in the regulation of SIRT3 expression by deacetylation and binding as a transcription factor (42). In addition, SIRT1 is involved in the deacetylation of retinoblastoma (Rb), leading to dissociation of E2F1 and enhanced cell proliferation (43). There might be a correlation among SIRT3, SIRT1, and pRB expression levels in HCC cells. Indeed, there was a negative correlation between SIRT1 and SIRT3 expression in data from patients with HCC from the TCGA

database (Supplementary Fig 1C). However, the mechanism of SIRT3 expression by CDK3/6 inhibition remains unclear. Thus, additional exploration will be needed in future studies.

Sorafenib has not been effective in patients with advanced HCC, and its use is often associated with reduction of drug sensitivity. Therefore, it is very important to identify a drug candidate that can replace or be used together with sorafenib. Tao *et al.* have found that upregulation of SIRT3 expression can enhance the sensitivity of HCC cells to chemotherapeutic agents (26). In our study, we found that the upregulation of SIRT3 by transfection in HCC cells reduced cell proliferation and significantly increased sensitivity to sorafenib. Moreover, the restoration of SIRT3 by PD0332991 could increase sensitivity to sorafenib, and their synergistic action reduced cell proliferation upon the combination of two drugs. Thus, we propose SIRT3 expression as a predictor of sorafenib response.

¹⁸F-PET-FDG is a surrogate imaging modality to measure glucose metabolism in patients with HCC. However, there are few studies on [¹⁸F] FDG-PET imaging in patients treated with sorafenib (44). Our results prove the negative correlation between the expression of SIRT3 and glucose metabolism using human HCC tissues and also HCC cells *in vitro*. Since the expression of SIRT3 is a predictor of response to sorafenib, [¹⁸F] FDG-PET imaging could monitor the drug sensitivity in HCC patients clinically during sorafenib treatment.

Conclusions

Taken together, our data indicate the importance of CDK4/6 inhibitors as a new approach to improve HCC therapy. Moreover, our study shows that induction of SIRT3 by CDK4/6 inhibition causes inhibition of cell growth, inhibition of glucose metabolism, and increased susceptibility to chemotherapy. Thus, the modulation of SIRT3 might be a novel treatment in patients with HCC and, possibly, with other cancers in which SIRT3 acts as a tumor suppressor.

Abbreviations

HCC: Hepatocellular carcinoma

RT-PCR : Real-time polymerase chain reaction;

FDG: ¹⁸F-fluorodeoxyglucose

PET/CT: positron emission tomography/computed tomography

TCGA: The Cancer Genome Atlas

CDK: Cyclin-dependent kinases

Declarations

Ethics approval and consent to participate

All the patients were informed of sample collection and usage. The tissue samples were collected and used in accordance with approval by the Clinical Research Ethics Committee of Yonsei medical university.

Consent for publication

The authors declare that they agree to submit the article for publication.

Availability of data and materials

All data analyzed during this study are included in this manuscript.

Competing interests

The authors declare that they have no competing interests

Funding

This research was supported by the National Research Foundation of Korea (Seoul, Korea; grant numbers NRF-2015R1D1A1A01057737, NRF-2016R1E1A1A01943303, and NRF-2018R1C1B6003894) and by an Incheon National University Research Grant (Incheon, Korea) in 2016.

Acknowledgements

Not applicable

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Figures

Figure 1

Figure 1

SIRT3 expression in patients with HCC and with different ^{18}F -FDG uptake. A. RNA was extracted from frozen hepatocellular carcinoma (HCC) samples obtained after transsphenoidal surgery. RT-PCR was performed using probe sets specific for SIRT3. The expression of the target genes was normalized to that of B2M (housekeeping gene) using the $2^{-\Delta\Delta\text{Ct}}$ method. The boundary of the box closest to zero indicates the 25th percentile, the line within the box marks the median, and the boundary of the box

farthest from zero indicates the 75th percentile. B. Formalin-fixed, paraffin-embedded (FFPE) human HCC samples were used. IF was performed using the indicated antibodies and counterstained with DAPI. Scale bars: 50 μ m, *, P < 0.05.

Figure 2

Figure 2

Expression of SIRT3 in hepatocellular carcinoma (HCC) cell lines and HCC xenograft models. A SIRT3 expression in four different HCC cell lines was measured using quantitative RT-PCR (qRT-PCR). The

expression level of target genes was normalized to that of the housekeeping gene B2M using the 2- $\Delta\Delta C_t$ method. Data are shown as the mean of three independent experiments $\pm$ SD. B Western blotting in different HCC cell lines using antibodies against SIRT3 and actin. C Formalin-fixed, paraffin-embedded (FFPE) liver tissues from HCC xenograft model were used. Immunohistochemistry was performed using antibodies against SIRT3, GLUT1, and Ki67 and counterstained with hematoxylin. Scale bars: 20 μ m. D. Huh7 cells were transfected with MOCK vector and pcDNA-SIRT3. After 48 h of incubation, protein was extracted and the expression of SIRT3, Ki67, and actin was determined using western blotting. E. In parallel, glucose uptake was measured using Glucose-Glo Assay. Data are shown as the mean of three independent experiments $\pm$ SD. **, P < 0.01.

Figure 3

A

B

C

Figure 3

Reduction of SIRT3 levels upon sorafenib treatment. A Reduced SIRT3 expression upon sorafenib treatment in hepatocellular carcinoma (HCC) cells. HCC cells were incubated with DMSO, 1 μ M sorafenib, or 10 μ M sorafenib. After 48 h, we measured the levels of SIRT3 and actin with western blotting. B Huh7 cells were transfected with MOCK or pcDNA-SIRT3. After 24 h, we treated 10 μ M sorafenib for further 24 h and then measured cell proliferation using WST-1 assays. Data are shown as the mean of three independent experiments $\pm$ SD. *, P < 0.05.

Figure 4

Figure 4

Correlation between SIRT3 and Rb. A Formalin-fixed, paraffin-embedded (FFPE) liver tissues from HCC xenograft model were used. Immunohistochemistry was performed using antibodies against phosphor-Rb (pRb) and counterstained with hematoxylin. Scale bars: 50 μ m. B RNA was extracted from frozen HCC samples obtained after transsphenoidal surgery. RT-PCR was performed using probe sets specific for Rb. The expression of the target genes was normalized to that of B2M (housekeeping gene) using the $2^{-\Delta\Delta C_t}$ method. The boundary of the box closest to zero indicates the 25th percentile, the line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. *, $P < 0.05$. C Formalin-fixed, paraffin-embedded (FFPE) liver tissues from human patients with HCC were used. Immunohistochemistry was performed using antibodies against SIRT3 (left) and pRb (right) and counterstained with hematoxylin. Scale bars: 50 μ m.

Figure 5

Figure 5

SIRT3 induction after PD0332991 treatment. A HepG2, Hep3B, SK-Hep1, and Huh7 cells were incubated with DMSO, 1 μM PD0332991, or 10 μM PD0332991. After 48 h, SIRT3 and actin levels were evaluated using western blotting. B HepG2 cells were transfected with scrambled siRNA oligos or siRNA oligos against CDK4/6. After 48 h, we performed western blotting to detect indicated proteins. C Fold change of

the indicated genes obtained by gene expression profiling of HepG2 cells after transfection with scrambled siRNA oligos or siRNA oligos targeting CDK4/6.

Figure 6

Figure 6

Increased sensitivity to sorafenib after combined treatment with sorafenib and PD0332991. A HepG2 cells were incubated with DMSO or 10 μ M PD0332991 with or w/o 1 μ M sorafenib. After 48 h, SIRT3 levels were analyzed using qRT-PCR. The expression level of target genes was normalized to that of the

housekeeping gene B2M using the $2^{-\Delta\Delta C_t}$ method. Data are shown as the mean of three independent experiments $\pm$ SD. B Cells were treated as in (A). Protein was extracted and the expression of SIRT3 and actin was determined using western blotting. C. In parallel, proliferation was assessed 48 h later using WST-1 assays. D Huh7 cells were incubated with DMSO, or 10 μ M PD0332991 with or w/o 1 μ M sorafenib. After 48 h, protein was extracted and the expression of SIRT3 and actin was determined using western blotting. E In parallel, proliferation was assessed 48 h later using WST-1 assays. Data from six technical replicates were analyzed and expressed as the mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Supplementary Files

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