Tumor suppressive role of sirtuin 4 in induction of G2/M arrest and apoptosis in HBV-related hepatocellular carcinoma

Huang FY[1], Wong DKH[1, 2], Seto WK[1, 2], Mak LY[1, 2], Yuen MF[1, 2]

[1]Department of Medicine, University of Hong Kong, Queen Mary Hospital, The University of Hong Kong, Hong Kong
[2]State Key Laboratory for Liver Research, The University of Hong Kong, Hong Kong

Abstract

The hepatitis B virus (HBV) HBx protein is associated with mitochondrial dysfunction and hepatocarcinogenesis. Recent studies suggested a tumor suppressor role of mitochondrial sirtuin 4 (SIRT4) in cancers. However, little is known about its effect on hepatocarcinogenesis. We aimed to investigate the clinical significance and functional role of SIRT4 in HBV-related HCC. SIRT4 expression was significantly downregulated in five HCC cancer cell-lines compared with two normal liver cell-lines (all $p<0.01$). Downregulation of SIRT4 was observed in human HCC tumor and adjacent non-tumor tissues compared with normal liver tissues (both $p<0.0001$). Analysis of TCGA data indicates SIRT4 levels were downregulated in patients with HBV infection but positively associated with better survival in patients with HCC. In vitro experiments revealed that stable HBx transfection suppressed SIRT4 expression in HepG2 and Huh7 cells (both $p<0.001$). Ectopic SIRT4 overexpression alone could induce cellular senescence through arresting cell-cycle progression at G2/M, and inducing cell apoptosis in HCC cancer cells. Mechanistically, SIRT4 upregulated cell-cycle governing genes p16 and p21 protein expression, suppressed CyclinB1/Cdc2 and Cdc25C which normally induce cell-cycle progression, and suppressed survivin to induce apoptosis. Our findings demonstrate the interaction between HBV and SIRT4 in the context of HCC. SIRT4 involves in G2/M DNA damage checkpoint control and genomic stability in hepatocarcinogenesis, which could be targeted for future anticancer strategies.

Introduction

- Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide.
- Chronic infection with hepatitis B virus (HBV) is a major risk factor for HCC.
- The hepatitis B X protein (HBx) encoded by the HBV X gene is associated with mitochondrial dysfunction and hepatocarcinogenesis.
- Mitochondrial sirtuin 4 (SIRT4) is a member of the sirtuin family (SIRT1-7) which involves in multiple cellular processes,
- Increasing evidence has indicated that SIRT4 functions as a mitochondrial-localized tumor suppressor in various cancers.
- Little is known about the interaction of HBx and SIRT4 in the context of HBV-related HCC.

Methodology

Tissues & cell lines

- 30 paired tumor (T) and non-tumor (NT) liver tissues were collected from patients who had HBV-related HCC.
- Control liver biopsies were obtained from 9 patients without HCC and HBV-infection.
- Cell lines were obtained from commercial sources and cultured in medium as suggested.

Gene expression analysis

- RNA and protein were extracted from the frozen tissue samples and cell lines
- mRNA expression of SIRT4 were measured by real-time PCR using GAPDH as an internal control.
- Gene expression of SIRT4 were further validated using TCGA datasets (Accession Nos: GSE39791, GSE22058, GSE25097, GSE36376, GSE46444, GSE54236 and GSE64041).
- Protein expression of the genes were assessed using Western-blot.

Plasmid transfection

- The HBx expression plasmid (pHBx) and SIRT4 plasmid were transfected into cell lines using lipofectamine 3000.
- Cells were selected by Geneticin for stable HBx transfection.
- Transfection was confirmed by qRT-PCR and Western blot analysis.

Cell growth, cell cycle and apoptosis analysis

- Cell growth of stable HBx transfected cells (HepG2-HBx and Huh7-HBx) were assessed using the CellTiter 96® Aqueous One solution.
- Cell cycle arrest and apoptosis of HepG2 and HepG2.2.15 with SIRT4 overexpression were measured using the flow cytometer.

Senescence-associated beta-galactosidase assay

- Cell senescence were determined in HepG2 and HepG2.2.15 cells with overexpression of SIRT4 using the Senescence-associated beta-galactosidase assay.

Statistical analysis

- Continuous variables were expressed as mean ± standard error of the mean (SEM) and analyzed using the student's t-test.
- Survival analysis was performed by Kaplan–Meier method, and the survival between groups was estimated with log-rank test.
- A p value of less than 0.05 was considered statistically significant.

Results

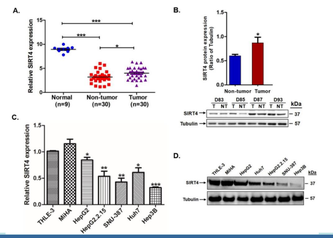

Figure 1: SIRT4 expression in liver tissues and cell-lines. (A) mRNA expression of SIRT4 in liver tissues. (B) SIRT4 protein expression in paired HCC samples. (C) SIRT4 mRNA expression in HCC cell-lines and normal liver cell-lines THLE-3 and MIHA. (D) Downregulation of SIRT4 protein expression in liver cancer-cell. (*** $p < 0.0001$, ** $p < 0.001$, *$p < 0.01$).

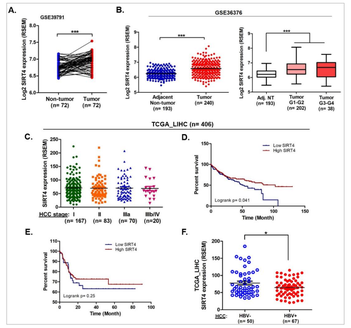

Figure 2: Confirmation of SIRT4 expression using TCGA datasets. (A-C) Lower SIRT4 levels in non-tumor than tumor tissues. (D-E) High SIRT4 levels correlate with better survival in HCC patients and patients without HBV infection. (F) SIRT4 expression was lower in patients with HBV infection. (*** $p < 0.0001$, *$p < 0.01$).

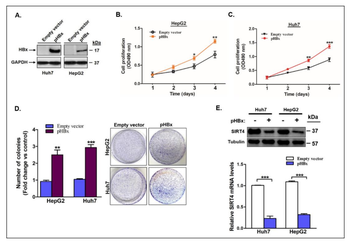

Figure 3: HBx induced HCC cell growth and suppressed SIRT4 expression. (A-D) HBx transfection induced cell growth and colony formation.(E) HBx transfection reduced SIRT4 expression. (*** $p < 0.0001$, ** $p < 0.001$, *$p < 0.01$).

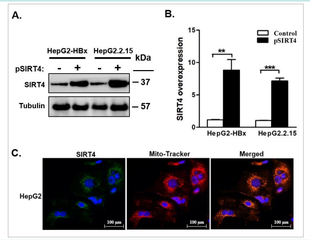

Figure 4: Overexpression of mitochondrial SIRT4 in cell-lines with stable HBx transfection. (A-B) Western blot and qRT-PCR confirm SIRT4 overexpression in HepG2-HBx and HepG2.2.15 cells. (C) Fluorescent images showing mitochondrial localization of SIRT4. (**$p < 0.01$, and ***$p < 0.001$).

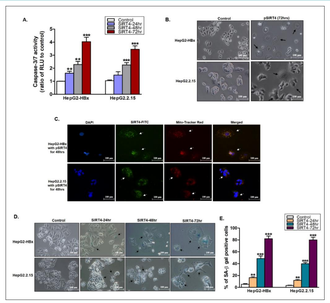

Figure 5: Effects of SIRT4 overexpression (A) Caspase3/7 activity increased in transfected cells. (B-C) Representative images showing SIRT4 overexpression induced cell senescence. (D) Representative images showing positive staining for SA-β-gal activity. Control cells show few or no staining. (E) Quantification of SA-β-gal positive cells Data are presented as mean ± SD of triplicate samples. (**$p < 0.001$, and ***$p < 0.0001$).

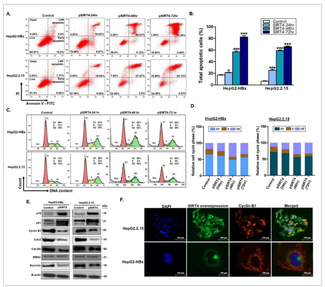

Figure 6: Effects of SIRT4 on cell-cycle progression. (A-B) Flow cytometry analysis showing apoptotic cells was increased with time. (C-D) SIRT4 overexpression induces cell-cycle arrest at G2/M. (E) Western blot-analysis of cell-cycle related proteins expression at 48hrs after SIRT4 transfection. (F) Representative fluorescence images showing the retention of Cyclin B1 in the cytoplasm after SIRT4 overexpression. (*$p < 0.01$, and ***$p < 0.0001$) .

Conclusions

- Our findings demonstrate that HBV infection has correlation with mitochondrial SIRT4 expression.
- The oncogenic HBx protein is associated with downregulation of SIRT4 expression. Activation of SIRT4 could induce cell apoptosis and G2/M arrest thus halt cancer cell cycle progression.
- Taken together, these findings suggest that SIRT4 might play a protective tumor suppressive role in HCC tumor initiation and promotion.
- Further in-depth study on the intrinsic molecular mechanism of SIRT4 might offer potential targeted genes for molecular therapy in HCC.

References

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