Histone deacetylase inhibitor suberoylanilide hydroxamic acid suppresses the pro-oncogenic effects induced by hepatitis B virus pre-S$_2$ mutant oncprotein and represents a potential chemopreventive agent in high-risk chronic HBV patients

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Chronic hepatitis B virus (HBV) infection is the major cause of hepatocellular carcinoma (HCC). The pre-S$_2$ mutant large HBV surface antigen (LHBS) in type II ground glass hepatocytes (GGHs) has been recognized as an emerging viral oncoprotein; it directly interacts with the c-Jun activation domain-binding protein 1 (JAB1) and subsequently causes hyperphosphorylation of the tumor-suppressor retinoblastoma and, consequently, leads to disturbed cell cycle progression. The interaction of the pre-S$_2$ mutant LHBS with JAB1 could provide a potential target for chemoprevention. In this study, we found that the preneoplastic type II GGHs showed a significant decrease of the cyclin-dependent kinase inhibitor p27$^{kip1}$, which serves as a marker for pre-S$_2$ mutant-JAB1 complex formation. The histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA) elevated expression of the tumor-suppressor thioecdoxin-binding protein 2 (TBP2), which subsequently enhanced the JAB1-TBP2 interaction and abolished the pre-S$_2$ mutant LHBS-induced degradation of p27$^{kip1}$, which, in turn, recovered the normal cell cycle checkpoint. The pre-S$_2$ mutant LHBS-induced pro-oncogenic effects: increased cell proliferation, nuclear/cytoplasmic ratio and proliferating cell nuclear antigen expression, were all greatly ameliorated after SAHA treatments, which suggested SAHA as a promising chemopreventive agent for the pre-S$_2$ mutant oncprotein-induced HCC. In conclusion, this study provides the mechanism of histone deacetylation (HDAC) inhibitor in preventing the pre-S$_2$ mutant-induced oncogenic phenotype. The HDAC inhibitor SAHA is therefore a potential chemopreventive agent for high-risk chronic HBV patients who may develop HCC.

Introduction

Chronic viral hepatitis is the major cause of hepatocellular carcinoma (HCC), the most frequent visceral neoplasm worldwide. The main causative agents for HCC are chronic hepatitis B virus (HBV) and HCV, which together are responsible for the majority of HCC in humans. These viruses cause necroinflammatory liver disease of variable duration and severity. A major portion of the viral hepatitis progresses into liver cirrhosis and dysplasia, and ultimately into HCC. In this process, the viral proteins are believed to be important players that cross talk with various host proteins, which affects the host signaling pathways. A number of HBV gene products have been identified as viral tumor proteins. The X protein (pX) is oncogenic because it activates a variety of carcinogenesis relevant cellular and viral promoters through direct binding to transcription factors and coactivators. HBX also stimulates the Src, Ras, NF-kB, and NF-AT signaling pathways (1–5). In addition, the HBV large surface antigen (LHBS) causes strong and sustained hepatocyte proliferation in the chronic phase of HBV infection in transgenic mice and is, therefore, highly associated with HCC incidence (6).

The large form of HBV surface (HBS) antigen, which includes an additional pre-S region that is the upstream promoter region for the small form, is predominantly expressed in the late phase of chronic HBV infection (7). In this stage, there emerges the pre-S$_2$ mutant LHBS that is truncated of ~17 amino acids in the N-terminus of the pre-S$_2$ region, and often also contains a point mutation in the start codon of the region, which leads to a dramatic decrease in the synthesis of the middle form of HBS (8,9). The pre-S$_2$ mutant LHBS contributes to the histological morphology of the type II GGH, which exhibited growth advantage and have been recognized as preneoplastic lesions (10,11). Therefore, the pre-S$_2$ mutant LHBS has been associated with hepatocarcinogenesis and represents a HBV viral oncoprotein (12).

Earlier studies (12,13) found that HBV carriers who presented with the pre-S$_2$ mutant LHBS in serum were predisposed to advanced liver diseases including liver cirrhosis and HCC. We found that the pre-S$_2$ mutant LHBS accumulates in endoplasmic reticulum (ER) and induces strong endoplasmic reticulum stress, which induces oxidative stress, DNA damage and mutagenesis, all of which cause genomic instability in hepatocytes (10,14). The pre-S$_2$ mutant LHBS also induces over-expression of cyclin A, which causes cell cycle progression in the presence of DNA lesions (15). We also recently found that the pre-S$_2$ mutant LHBS directly interacts with c-Jun activation domain-binding protein 1 (JAB1) and subsequently causes hyperphosphorylation of the tumor-suppressor retinoblastoma and, consequently, G1 to S phase cell cycle progression (16). JAB1 is a key subunit of the COP9 signalosome and acts as a multifunctional protein associated with the signaling pathway, cell cycle regulation and development (16). JAB1 promotes cell proliferation by activating AP-1 transcription and stimulates cell cycle progression by increasing the degradation of the cyclin-dependent kinase inhibitor p27$^{kip1}$ (17). The interaction of the pre-S$_2$ mutant LHBS with JAB1 is, therefore, an important mechanism for the carcinogenic process associated with it. Intrusion to disrupt the pre-S$_2$ mutant-JAB1 complex using a chemical method could potentially provide a chemopreventive approach to inhibit the pro-oncogenic property contributed by the pre-S$_2$ mutant LHBS.

HBV infection-associated HCC is usually multifocal and therefore difficult to surgically eradicate. Patients who received surgical resection had high recurrence rates. Our earlier study found that the type II ground glass hepatocytes (GGHs) harboring pre-S$_2$ mutant LHBS represent preneoplastic lesions preceding HCC (10,11). Recently, we also found that the presence of type II GGHs predicts the recurrence of HCC after surgery (18). The type II GGHs are therefore high-risk markers for HCC in chronic HBV carriers and recurrence after hepatectomy surgery. Because HBV-induced HCC usually develops many years after the initial infection, it allows time for a chronic HBV carrier to uptake a chemopreventive approach, which prevents HBV($^{e}$) hepatocyte transformation and carcinogenesis (19).

Abbreviations: a.a., amino acid; GGH, ground glass hepatocyte; HBS, HBV surface; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; HRP, horse radish peroxidase; i.p., intraperitoneally; JAB1, c-Jun activation domain-binding protein 1; KD, knock down; LHBS, large HBV surface antigen; RT-PCR, reverse transcriptase PCR; siRNA, small interfering RNA; SAHA, suberoylanilide hydroxamic acid; TBP2, thioecdoxin-binding protein 2.

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Pre-S mutant LHBS is highly prevalent in the late phase of chronic HBV infection and HCC, and the hepatocytes harboring it stand out as those most susceptible to cancer (20). Therefore, developing a chemopreventive approach that targets these cells is important.

JAB1 interacts with the tumor-suppressor thioredoxin-binding protein 2 (TB2p), which forms a competitive binding with the JAB1-p27Kip1 complex and stabilizes p27Kip1 (21). We, therefore, hypothesized that TB2p could serve as a molecular target for p27 stabilization. A previous study reported that the histone deacetylase (HDAC) inhibitor suberylanilide hydroxamic acid (SAHA) increased TB2p in human prostate and bladder cancer cells (22). HDAC inhibitors have also been well known to present with a wide substrate spectrum, including various HDACs, p53, c-Myc, etc. (23). The concerted actions of HDAC inhibitors on these molecules inhibit tumor cell proliferation and division; whereas they mostly cause little harm to non-cancerous cells (23). SAHA, a potent HDAC inhibitor, has been approved for treating some cancers, such as cutaneous T-cell lymphoma (24). In this study, we studied the molecular mechanism of how SAHA regulates TB2p levels in liver cells. We also examined whether by increasing the TB2p level, SAHA could recover the normal p27(Kip1) level that was aberrantly lowered by pre-S(S2) mutant LHBS.

This allowed us to investigate the potential of SAHA as a chemopreventive agent for high-risk chronic HBV patients who may develop HCC or whose HCC may recur after surgery.

Materials and methods

HCC patients and cell lines

Non-tumorous liver sections were collected with informed consent from the HCC patients admitted to National Cheng Kung University Hospital, for hepatectomy surgery from 2005 to 2009. Human hepatoma HuH7, immortalized non-cancerous hepatocyte WRL-68, and bladder cancer T24 cell lines were used for in vitro cell culture studies. These cells were transiently transfected with the wild-type and pre-S(S2) mutant LHBS, cloned in the pRES-hrGFP-2a plasmid vector (Stratagene), which contained the hemagglutinin epitope (HA).

The pre-S gene chip

The protocols for detecting pre-S gene deletions using the pre-S gene chip, developed in our laboratory, are described previously (20). Briefly, the pre-S(S2) mutant LHBS was PCR amplified by using the 5′-digoxigenin-labeled primers then incubated with a pre-S gene chip in a 24-well culture plate in microarray hybridization buffer [5× saline sodium citrate, 1% blocking reagent (Roche), 0.1% N-lauroylsarcosine, 0.02% sodium deoxydetyl sulfate (SDS)]. The digoxigenin that had hybridized to the DNA probes on the chip was then recognized by incubating the chip with the anti-digoxigenin-alkaline phosphatase Fab body and protein A/G agarose beads (Santa Cruz Biotechnology) were mixed with the DNA–protein complexes. The underlined regions indicate the NF-Y-binding sites. To perform DAPA, cell nuclear proteins were incubated with biotinylated double-stranded probes at 37ºC for 2 h. The DNA–protein complexes were then pulled down using streptavidin-M of SAHA (Cayman Chemicals) for 24 h. The LHBS was detected using the mouse monoclonal antibody recognizing HA, tagged to the LHBS gene.

Luciferase reporter assays for TB2p promoter activities

HuH7 cells were transfected with the wild-type, pre-S(S2) mutant LHBS or the pGL-3 luciferase reporter plasmids carrying various TB2p promoter regions (22). After transfections for 24 h, the culture media were replaced with fresh media containing 5 µM of SAHA or vehicle [dimethyl sulfoxide (DMSO)]. After incubation for another 24 h, the cells were harvested and the luciferase activities were measured using Luciferase Assay System (Promega).

DNA affinity purification assay (DAPA)

Oligonucleotides containing the two putative nuclear transcription factor Y (NF-Y)-binding sites at the −450 and −415 promoter regions of TB2p gene were synthesized and biotinylated. Their complementary oligonucleotides were also synthesized then annealed to them as probes for DNA affinity purification assay (DAPA) analysis. The wild-type and mutant probes used were wild-type (−450 region): 5′-aggacgcccagccatctgag, mutant (−415 region): 5′-aggacgcccagccatctgag, and mutant (−415 region): 5′-acactccctccctgctgag, and mutant (−415 region): 5′-acactccctccctgctgag. The underlined regions indicate the NF-Y-binding sites. To perform DAPA, cell nuclear proteins were incubated with biotinylated double-stranded probes at 37°C for 2 h. The DNA–protein complexes were then pulled down using streptavidin beads (GenScript) then analyzed by western blotting using NF-YA antibody (GenScript).

Cell cycle analysis

Protocols for cell cycle analysis are described elsewhere (17). HuH7, T24 and WRL-68 cells stably expressing wild-type or pre-S(S2) mutant LHBS were treated with 5 µM of SAHA for 24 h then analyzed for cell cycle profiles. To detect cell cycle profiles, cells were harvested by trypsinization, washed twice with phosphate-buffered saline (pH 7.4), fixed with ice-cold 70% ethanol overnight, and then stained with propidium iodine (40 µg/ml) supplemented with RNAse A (0.1 mg/ml) for 10 min at room temperature. The cell cycle profiles were detected by FACScan flow cytometry (Becton Dickinson). The percentages of cells in the G1, S and G2/M phases were measured by using the WinMDI software.

Colony formation assays

One thousand HuH7 or WRL-68 cells stably expressing wild-type or pre-S(S2) mutant LHBS were seeded in a 10 cm cell culture plate with growth medium containing SAHA (1 µM) or the vehicle control DMSO. The cells were maintained for 2 weeks, and growth medium was changed every 3 days. The surviving cell colonies were then stained with crystal violet (1%) and counted.
**Pre-S2 mutant LHBS transgenic mice**

The transgenic and the control C57BL/6 mice were maintained in the animal facility in National Cheng Kung University medical center and fed standard laboratory chow and water ad libitum based on Taiwan National Science Council guidelines. Sera from these mice were routinely examined for ALT levels (GPT/ALT-III kit; Fujifilm). Liver sections taken from six transgenic and six control C57BL/6 mice at 6-months old were examined for LHBS expression levels using western blotting with a mouse antibody targeting the pre-S2 region (generously provided by Tsai-Ming Yeh, PhD, National Cheng Kung University). Western blotting was also used to detect other proteins in mouse livers (15).

**SAHA treatment of the pre-S2 mutant LHBS transgenic mice**

Five 6-month-old pre-S2 mutant LHBS transgenic mice and five age- and sex-matched C57BL/6 control mice were intraperitoneally (i.p.) injected with SAHA (50 mg/ml/day) or vehicle (33.3% DMSO) and 66.7% of 0.01 M phosphate buffer (pH 7.4) for 14 days before they were euthanized.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assays**

Five micrometer paraffin-embedded liver sections were selected for apoptosis using the TUNEL Detection Kit (TACS TdT; R&D Systems).

**Results**

**Pre-S2 mutant LHBS-mediated p27Kip1 degradation in type II GGHs of HCC patients**

To specifically detect the pre-S gene deletions in GGH nodules, we did immunochemical staining for HBS antigen in the non-tumorous sections of the HCC tissue samples. Type I GGHs, sporadically scattered, and type II GGHs, clustered as nodules, were observed (Figure 1A, Supplementary Figure 1, available at Carcinogenesis Online). An analysis, using Pre-S Gene Chips, of isolated morphologically normal hepatocytes and GGHs showed that type I and type II GGHs harbored deletions in the pre-S1 and -S2 regions, respectively, and that normal hepatocytes did not express pre-S mutant LHBS, which showed a high correlation between GGH morphology and pre-S deletions (Figure 1B). p27Kip1 had been found degraded by pre-S2 mutant LHBS in cell cultures in vitro (17). Immunofluorescence analysis of p27Kip1 in the sections containing type I and type II GGHs showed that type II GGHs (clumped) had lower p27Kip1 levels than type I GGHs (sporadic) or the hepatocytes that did not harbor HBS antigen (Figure 1C, Supplementary Figure 2, available at Carcinogenesis Online). In total, 84.6% (11 of 13) of the type II GGH sections and none (0 of 9) of the type I GGH sections examined showed a decrease in p27Kip1. This finding indicates that a p27Kip1 decrease is specifically prevalent in the type II GGH cells in HCC patients and can potentially serve as a biomarker for pre-S2 mutant LHBS-induced HCC.

**Requirement of amino acids 61–119 in pre-S2 mutant oncoprotein for JAB1-induced p27Kip1 degradation in GGHs**

To characterize the protein interactions between JAB1 and pre-S2 mutant oncoprotein, we mapped the pre-S2 mutant LHBS domain that directly binds to JAB1 using yeast two-hybrid assays. Consistent with our earlier findings (17), the pre-S2 mutant LHBS binds to JAB1, as the yeast cells carrying these two proteins expressed the Gal4 promoter-driven histidine reporter gene and grew in the histidine-selective medium (Figure 2A). Analyses of the serially truncated pre-S2 mutant LHBS constructs found that the truncated proteins maintained their affinities to JAB1, except the one truncated in the region spanning amino acid (a.a.) 61–119, which indicated that a.a. 61–119 was the interactive domain for binding to JAB1. The proteins truncated in the regions spanning a.a. 61–90 and a.a. 91–119 lost most of their abilities to bind with JAB1, which showed that the interactive domain spans a.a. 61–119 (Figure 2B). Amino acid 61–119 is in the pre-S2 promoter region; therefore, the pre-S2 mutant LHBS is associated with JAB1 through the pre-S2 region, and this is specific to the large form of HBS (Figure 2C). Requirement of this domain for the interaction between the two proteins in vivo was also confirmed using co-immunoprecipitation assays, which showed that, unlike the full-length protein, the pre-S2 mutant LHBS truncated in this domain was not associated with JAB1 (Figure 2D). We previously reported that pre-S2 mutant LHBS induced JAB1-dependent p27Kip1 degradation and disturbed cell cycle checkpoints in transgenic mice (17). In this study, we found that this effect was dependent on the interaction between JAB1 and pre-S2 mutant LHBS, because the p27Kip1 level in the truncated protein (Δ264–119; Figure 2E), which was no longer bound to JAB1, recovered to that in the control cells (V; Figure 2E), which contained vehicle plasmid only. MG132, a 26S proteasome inhibitor, was added to the pre-S2 mutant LHBS(+)-cells to show that the observed p27Kip1 decrease was indeed caused by the proteasome-mediated degradation (Δ2 versus Δ2 w/MG132, Figure 2E). We previously reported that pre-S2 mutant LHBS caused phosphorylation on threonine 160 residue of the G1 to S phase cell cycle regulator Cdk2 as a result of p27Kip1 degradation (17). In this study, we found that in the cells carrying truncated pre-S2 mutant LHBS (Δ264–119; Figure 2E), the Cdk2 phosphorylation on the threonine 160 residue was greatly inhibited (Δ2 versus Δ2Δ264–119 in the panel of CDK2 (Figure 2E). These results clearly demonstrated that the a.a. 61–119 region of the pre-S2 mutant LHBS was essential for JAB1-mediated p27Kip1 degradation in type II GGHs.

**SAHA induced TBP2-mediated p27Kip1 recovery in pre-S2 mutant hepatocytes**

It was previously reported that the HDAC inhibitor SAHA induced expression of TBP2, which binds to p27Kip1 (22). We, therefore, hypothesized that, by increasing the TBP2 level with an HDAC inhibitor, the level of p27Kip1 would recover to a normal state in pre-S2 mutant LHBS(-) hepatocytes. Indeed, in the pre-S2 mutant LHBS(-) Huh7 cells treated with SAHA, we found that the TBP2 RNA and protein levels were both greatly increased, concordantly with p27Kip1 protein increase (Figure 3A and B), indicating that TBP2 was transcriptionally upregulated by SAHA. Some pre-S2 mutant LHBS-induced oncogenic effects: cyclin A overexpression, Cdk2(160)-phosphorylated retinoblastoma hyperphosphorylation, reported by us, were greatly inhibited after SAHA treatments (Figure 3A and B). It was reported (22) that SAHA activated TBP2 transcription through the nucleotide –482 to –264 promoter regions of the gene, which contains two putative binding sites to NF-Y and one to Sp1 transcription factors. We found that, mutation in either of the two NF-Y binding sites dramatically inhibited the SAHA-induced TBP2 transcriptional activation, and mutations in both sites nearly completely abolished this effect (Figure 3C). However, mutation in the Sp1 binding site only mildly inhibited this effect (Figure 3C). These findings suggested that NF-Y is the main transcription factor regulating SAHA-induced TBP2 expression. By DAPA analysis, it was found that SAHA treatments greatly increased the binding of NF-Y to both oligonucleotide probes containing the either NF-Y binding sites but not the ones where the NF-Y binding sites were mutated (Figure 3D). These findings revealed that SAHA-induced TBP2 expression through enhancing NF-Y transcription factor binding to the gene promoter.

We also found that, in the pre-S2 mutant LHBS(-) cells treated with SAHA, the knockdown of TBP2 expression with a specific siRNA brought the levels of p27Kip1 protein and Cdk2(160)-phosphorylation back to those before SAHA treatment (Figure 3E), indicating that inhibiting HDAC activities efficiently recovered the normal functions of the cell cycle regulators p27Kip1 and Cdk2 through a TBP2-dependent pathway. Co-immunoprecipitation assays showed that SAHA increased the association of JAB1 with TBP2 (Figure 3F). Also, to precisely detect the binding of JAB1 with p27Kip1, cells were treated with the 26S proteasome inhibitor MG132 to block p27Kip1 degradation, and then treated with SAHA or otherwise the solvent DMSO. It was found that SAHA decreased the JAB1-p27Kip1 interaction, which was aberrantly high in pre-S2 mutant LHBS(-) cells (Δ2 w/SAHA versus Δ2 w/DMSO; Figure 3G). Taking these findings together, through its activity as an HDAC inhibitor, SAHA restored the normal G1 to S cell cycle checkpoint that was disrupted by pre-S2 mutant LHBS.

**SAHA restored normal cell cycle checkpoints and proliferation hindered by pre-S2 mutant LHBS**

Cell cycle profile analysis found that pre-S2 mutant LHBS(-) cancerous T24 cells and non-cancerous WRL-68 hepatocytes showed...
Fig. 1. P27Kip1 degradation and pre-S deletions in the GGHs of a non-tumorous tissue sample from a patient with HCC. (A) The HBS IHC staining results for one representative tissue sample from a patient with HCC. The morphologically normal cells and GGHs in the non-tumorous tissue were magnified to 200× and are shown in separate images. Red arrowheads point to the regions in the tissue where the images were captured. (B) Summary of the pre-S deletions in the normal and GGH tissue samples, detected using Pre-S Gene Chips. ND, not detectable, no HBS gene was detected. GGHII, type II GGH; GGHI, type I GGH. (C) Representative results of the immunofluorescent analysis to detect p27Kip1 in the type I and type II GGH tissue samples. The dotted white lines indicate the boundary of the type II GGH nodules. The HBS IHC results from the same regions in the paraffin-fixed tissue samples are shown to the right of the corresponding immunofluorescence images as references for the GGH morphologies.
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Fig. 2. The pre-S2 mutant LHBS protein domain involved in the interaction with JAB1. (A) Yeast two-hybrid assays to detect the interaction between the pre-S2 mutant LHBS and JAB1. The yeast cells transformed with the pre-S2 mutant LHBS gene in the GAL-BD-containing pGBK7 plasmid and JAB1 in the GAL-AD-containing pACT2 plasmid or vector plasmid-only were plated on both of the histidine-permissive and histidine-selective growth plates. Only the yeast containing both pre-S2 mutant LHBS and JAB1 were able to grow in the histidine-selective growth medium. –H and +H, histidine-selective and histidine-permissive growth media, respectively. (B) The yeast cells transformed with the various truncated pre-S2 mutant LHBS genes in the GAL-BD-containing pGBK7 plasmid and JAB1 in the GAL-AD-containing pACT2 plasmid or vector plasmid-only were tested for a histidine prototrophic phenotype. The pre-S2 mutant LHBS deleted from the protein regions spanning a.a. 1–119 (∆1–119), a.a. 61–119 (∆1–119), a.a. 61–90 (∆61–90) and a.a. 91–119 (∆91–119) lost interaction with JAB1, which indicated that a.a. 61–119 is the minimal interaction domain. a.a., amino acid; FL, full-length pre-S2 mutant LHBS. (C) Summary of the yeast two-hybrid experimental results. Numbers on the top of the schematic representation of the pre-S2 mutant LHBS indicate the amino acid positions at the C-terminal ends of the pre-S1 and pre-S2 regions. TM, transmembrane. (D) Co-immunoprecipitation assays to detect the interaction between pre-S2 mutant LHBS and JAB in HuH7 cells. Cell lysates of the HuH7 cells transiently transfected with wild-type (WT) LHBS, pre-S2 mutant LHBS (∆2), or that deleted from the region spanning a.a. 61–119 (∆2Δ61–119), or vector plasmid-only (V) were subjected to immunoprecipitation using anti-HA antibody, followed by western blotting. (E) The levels of p27\(^{\text{Kip1}}\), CDK2 and CDK2\(^{T160}\) phosphorylation in HuH7 cells transfected with full-length and truncated pre-S2 mutant LHBS, detected using western blotting. Lane 5, cells pretreated with the proteasome inhibitor MG132 (10 µM) for 4 h before harvest. α-Tubulin was the internal control.
Fig. 3. SAHA blocked p27Kip1 degradation by inducing TBP2 gene expression. (A) RT–PCR to detect the levels of TBP2, cyclin A and p27 mRNA in HuH7 cells treated with DMSO for 24 h (C) or SAHA (5 µM) for 4, 8, 12 and 24 h before analysis. β-Actin was used as loading control. (B) Western blotting to detect the levels of p27Kip1, TBP2, cyclin A and Cdk2\(^{T160}\) and retinoblastoma phosphorylations in HuH7 cells treated with DMSO for 24 h (C) or SAHA (5 µM) for 6, 12 and 24 h before analysis. Acetyl-histone H3 was used as a marker for HDAC activities after the treatments. Quantitative results of the western blotting from at least three independent experiments are shown at the bottom of the gel images. The intensity of each protein band was quantified and then normalized with that of β-actin in cells with the same treatment. The averaged values (±SD) of the results in the same group are indicated. (C) Luciferase reporter assays to detect the TBP2 promoter activities after SAHA (5 µM) treatments. The wild-type and mutant TBP2 promoter region nucleotide –482 to –264, where the two NF-Y (–450 and –415) and one SP1 (–423) binding sites were located, were cloned into the pGL-3 luciferase reporter plasmid and then transfected into HuH7 cells, which were then treated with SAHA (5 µM) or vehicle control DMSO. The mutated NF-Y and SP1 binding sites in each promoter construct were labeled with crosses (×). The relative luciferase activity was indicated by the fold increase in luciferase activity from that in the cells treated with DMSO to that in the cells transfected with the same plasmids but treated with SAHA. (D) DAPA analysis to detect the binding of NF-Y to the TBP2 promoter. Biotinylated DNA probes containing the wild-type (WT) or mutant (M) NF-Y binding sites were mixed with nuclear extracts of HuH7 cells that were pretreated with SAHA (5 µM) or DMSO, and
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Discussion

To prevent the disease progression from chronic HBV infection to HCC still remains a major challenge. Previously, we identified that type II GGHs harboring the pre-S mutant LHBS represent the preneoplastic lesions and provide a biomarker to identify the high-risk patients. In this study, we found the decrease of p27\(^{\text{Kip1}}\) in the type II GGHs in HCC patients via the interaction between the pre-S mutant LHBS and JAB1, suggesting p27\(^{\text{Kip1}}\) degradation as a biomarker for chronic HBV carriers in high risk to HCC. We also found that HDAC inhibitor SAHA increased transcription of the tumor suppressor TP2 through facilitating binding of the transcription factor NF-Y to TP2 promoter, which, in turn, increased the TP2-JAB1 complex. Through this pathway, SAHA was able to attenuate the pre-S mutant LHBS-induced JAB1-p27\(^{\text{Kip1}}\) interaction and recover normal cell cycle checkpoint disturbed by such an interaction. This effect was observed in \textit{in vitro} cultured cells and \textit{in vivo} pre-S\(_2\) mutant transgenic mice, which exhibited pro-oncogenic phenotypes including large cell dysplasia and enhanced proliferation. Data summarized in Figure 6 show that SAHA reduced PCNA overexpression and p27\(^{\text{Kip1}}\) degradation, which inhibited pre-S\(_2\) mutant LHBS-induced cell proliferation and cell cycle progression. Earlier studies (16,27–29) also showed that, as an HDAC inhibitor, SAHA abolished cyclin A overexpression and Akt/mTOR and vascular endothelial growth factor activation, all of which were found to be stimulated by the pre-S\(_2\) mutant LHBS in our earlier studies. Taking all these findings together, we conclude that SAHA is a promising potential chemopreventive agent for HCC in chronic HBV carriers harboring type II GGH preneoplastic lesions. A recent study has also reported that the ubiquitin ligase S-phase kinase-associated protein 2 (SKP2) were commonly increased in HCC and resulted in p27\(^{\text{Kip1}}\) degradation (30). In this study, we examined the SKP2 mRNA and protein levels in the human HuH7 cells carrying the pre-S\(_2\) mutant LHBS but observed no significant changes in them (Supplementary Figure 5, available at Carcinogenesis Online). This finding indicates that the pre-S\(_2\) mutant LHBS-induced p27\(^{\text{Kip1}}\) degradation is mainly through its interaction with JAB1 rather than SKP2-mediated ubiquitination activity.

Recent studies have reported that JAB1 overexpression and p27\(^{\text{Kip1}}\) degradation were commonly seen in HCC, suggesting that they were attractive molecular targets for HCC therapy and chemoprevention of recurrence after hepatectomy treatments (31,32). In this study, we also observed p27\(^{\text{Kip1}}\) degradation in type II GGHs harboring preneoplastic lesions and found that HDAC inhibitor SAHA caused p27\(^{\text{Kip1}}\) stabilization through inducing TP2 expression to disrupt JAB1-p27\(^{\text{Kip1}}\) complex. Therefore, it is suggested that the liver tissues obtained from the HCC patients receiving hepatectomy surgery and chronic HBV carriers receiving liver biopsy procedures should be examined for p27\(^{\text{Kip1}}\) level in order to decide whether they are suitable for chemoprevention using SAHA. Recently, SAHA and another HDAC inhibitor sodium butyrate were shown to exhibit chemopreventive activities for lung and colon cancers through epigenetically regulating some carcinogenesis-associated genes (33,34). These findings were consistent with ours in HBV-induced HCC. HDAC inhibitors are responsible for modifying the activity of diverse types of histone and non-histone proteins, including transcription factors and signal transduction mediators that possess tumor-associated properties (23). They downregulate the expression of oncopgenic genes, such as TGF-\(\beta\), telomerase, vascular endothelial growth factor, cyclin A and \(\alpha\)I genes (27–29). In
contrast, they also upregulate the expression of the tumor-suppressive
genes, such as p53, p21, gelsolin and a group of proapoptotic genes,
such as Bcl2, Bim and caspase 3 (23). In this study, we found that
SAHA increased direct binding of the transcription factor NF-Y to
the promoter of the tumor-suppressor TBP2, an upstream inhibitor of
thioredoxin, which presents with oncogenic properties through affect-
ing reduction–oxidation homeostasis and inhibiting apoptosis (35).
Previous studies have shown that the function of NF-Y as a transcrip-
tion factor was activated by acetylation, suggesting that SAHA might
have induced NF-Y acetylation and, consequently, its activation (36).
It was also reported that in HDAC inhibitor-induced apoptosis, the
production of proapoptotic reactive oxygen species was important
(37). We previously (15) found that the pre-S2 mutant LHBS induced
reactive oxygen species generation, which might augment the killing
effects of HDAC inhibitors on cells and explain why SAHA killed
non-cancerous WRL-68 cells carrying the pre-S2 mutant LHBS more
efficiently than those carrying the wild-type LHBS (Figure 4C).
Interestingly, we found that in the transgenic mice SAHA was able
to decrease the proliferation marker PCNA without causing signifi-
cant toxicity to the liver, indicated by negative results of the TUNEL
assays (Supplementary Figure 3, available at Carcinogenesis Online).
This finding suggested that SAHA affected the functions of its target
molecules, but was also efficiently metabolized and detoxicated via
the strong xenobiotic metabolizing activity in the liver.
HCC caused by chronic HBV infection is usually a slow process
with an incubation period of >20 years. An active chemopreventive
approach should therefore be undertaken in chronic HBV carriers
during the long years of viral persistence, especially in those that
represent high-risk populations. So far, most of the reported chem-
opreventive agents for HCC are phytochemicals presenting general
antioxidant activities to defeat the oxidative stress caused by hepatic
inflammation and necrosis (26,38,39). Common examples are chloro-
phylin, silymarin, vitamin E and vitamin A and its derivatives, such
as polyphenolic acid. However, it has been found that although these
chemicals prevented HCC carcinogenesis if administered at the early
stage before the initiation of the carcinogenic process, some of them
had no protective effects once pre-dysplastic phenotypes had emerged
(26). In fact, antioxidant activities in cancers have been found to
increase cancer cell proliferation, resulting in resistance to chemo-
therapies (40). HDAC inhibitor, however, is different in that it has
a highly selective killing effect on cancerous rather than on healthy
cells, and it has not been found to promote the growth of pre-dys-
plastic cells (23,41). In this study, we showed that HDAC inhibitor
SAHA inhibited proliferation of pre-S2 mutant LHBS* cells and non-cancerous cells, indicating that SAHA not only kills HCC
cells but that it also selectively eradicates pre-S2 mutant LHBS* non-
cancerous cells, which are prone to cancer. Given that, HDAC inhibi-
tor stands as a good potential preventive agent for HCC and it should
be particularly beneficial to the cells exhibiting high pro-oncogenic
phenotypes.
Previous and our current studies have found that some HDAC
inhibitors exhibited chemopreventive activities for cancer. Thus,
to be widely applied to large population of healthy HBV carriers
in daily base, using food-derived natural compounds would be
a practical way to chemically prevent HCC in healthy HBV car-
riers. Our preliminary data found that the natural HDAC inhibitor
sulforaphane, extracted from broccoli, selectively killed cancerous
cell (DMSO) for 24h and then harvested for propidium iodide staining,
followed by flow cytometric analysis. Data in the bar chart indicate the
averaged values (±SD) of at least three independent experiments. *p < 0.05
and **p < 0.001. (C) Colony formation assays to detect cell prolifera-
tion rates affected by SAHA. One thousand T24, HuH7 and WRL-68
cells stably transfected with wild-type (WT) LHBS, pre-S2 mutant (Δ2)
LHBS or vehicle control (V) were plated and treated with low-dose SAHA (1 µM) or vehicle
control (DMSO) for 2 weeks. The surviving colonies were stained with
crystal violet and then counted. Data in the bar chart indicate the averaged
values (±SD) of at least three independent experiments. **p < 0.05.
Histone deacetylase inhibitor SAHA suppresses the pro-oncogenic effects

Fig. 5. SAHA inhibited the pro-oncogenic phenotypes in livers of pre-S2 mutant LHBS transgenic mice. (A) Hematoxylin-eosin and (B and C) immunohistochemical stainings of HBS (B) and PCNA (C). Ctrl, C57BL/6 control. Δ2Tg, pre-S2 mutant LHBS transgenic mice. Scale bar = 100 µm. (D) Western blotting to detect the levels of p27Kip1, Cdk2 and Cdk2T160 phosphorylation in the liver protein extracts from 6-month-old control (Ctrl) and from pre-S2 mutant LHBS transgenic (Δ2Tg) mice. The results are representative of two mice in each group. Quantitative results of the western blotting from six mice in each group are shown at the bottom of the gel images. The intensity of each protein band was quantified and then normalized with that of β-actin in the same mouse. The averaged values (±SD) of the results from six mice in a group are indicated. **p < 0.05. (E–H) Five pairs of 6-month-old control (Ctrl) and pre-S2 mutant LHBS transgenic (Δ2Tg) mice were injected with SAHA (50 mg/ml/day; i.p.) or DMSO for 2 weeks and then euthanized for analysis. (E) Hematoxylin-eosin (HE) stain and immunohistochemical staining for HBS. Scale bar: 200 µm. (F) The mRNA levels of the p27Kip1, TBP2, PCNA, Ki67 and LHBS genes are detected using RT–PCR. The intensity of each PCR band on gel was quantified and then normalized with that of the β-actin gene in the same mouse. Data in the bar chart below the gel images indicate the averaged values (±SD) from five mice in each group. **p < 0.05. (G) The levels of the p27Kip1, TBP2, Cdk2T160 phosphorylation and LHBS genes are detected using western blotting. The intensity of each protein band was quantified and then normalized with that of the β-actin gene in the same mouse. Data in the bar chart below the western blotting results indicate the averaged values (±SD) from five mice in each group. **p < 0.05. (H) Cell proliferation rates are detected using PCNA immunohistochemical staining. The cells displaying strong positive (S), weak positive (W) and negative (N) signals of PCNA were quantified and indicated below. **p < 0.05.
Fig. 5. Continued

Fig. 6. Schematic representation for the anticarcinogenic effects of SAHA on pre-S2 mutant LHBS-induced HCC. The pre-S2 mutant LHBS induces endoplasmic reticulum stress-mediated oxidative stress and DNA damage that cause gene mutations and genomic instability. It also induces cyclin A overexpression and p27Kip1 degradation, which causes cell cycle progression. In addition, we recently showed that the pre-S2 mutant LHBS activates the Akt/mTOR proliferative signaling pathway and increases cell proliferation. These multiple effects greatly accelerate the hepatocellular carcinogenic process in chronic HBV carriers and indicate that the pre-S2 mutant LHBS is a significant viral oncoprotein. The concerted activities of SAHA to block cyclin A and PCNA expression, Akt/mTOR pathway activation and p27Kip1 degradation in a JAB1-dependent manner support the hypothesis that SAHA induces significant remission of pre-S2 mutant LHBS-induced oncogenic phenotypes and will, therefore, be an effective chemopreventive against pre-S2 mutant LHBS-associated HCC.

Supplementary material

Supplementary and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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