HMDB and 5-AzadC Combination Reverses Tumor Suppressor CCAAT/Enhancer-Binding Protein Delta to Strengthen the Death of Liver Cancer Cells

Chien-Feng Li¹, Hsin-Hwa Tsai², Chiung-Yuan Ko³, Yen-Chun Pan⁴, Chia-Jui Yen⁵, Hong-Yue Lai⁶, Chiou-Hwa Yuh⁷, Wan-Chen Wu⁴, and Ju-Ming Wang²,⁸

Abstract

Hepatocellular carcinoma (HCC) can arise from chronic inflammation due to viral infection, organ damage, drug toxicity, or alcohol abuse. Moreover, gene desensitization via aberrant CpG island methylation is a frequent epigenetic defect in HCC. However, the details of how inflammation is linked with epigenetic-mediated desensitization of tumor suppressor genes remains less investigated. In this study, we found that loss of CEBPD enhances the growth of liver cancer cells and is associated with the occurrence of liver cancers, as determined by the assessment of clinical specimens and in vivo animal models. Moreover, E2F1-regulated epigenetic axis attenuated CEBPD expression in liver cancer cells. CEBPD is responsive to the hydroxymethyl dibenzoylmethane (HMDB)-induced p38/CREB pathway and plays an important role in the HMDB-induced apoptosis of cancer cells. Regarding depression of epigenetic effects to enhance HMDB-induced CEBPD expression, the combination of HMDB and 5-Aza-2′-deoxycytidine (5-AzadC) could enhance the death of liver cancer cells and reduce the tumor formation of HuH7 xenograft mice. In conclusion, these results suggest that CEBPD could be a useful diagnostic marker and therapeutic target in HCC. The results also reveal the therapeutic potential for low-dose 5-AzadC to enhance the HMDB-induced death of HCC cells. Mol Cancer Ther; 14(11): 2623–33. ©2015 AACR.

Introduction

Epidemiologic studies suggest that chronic inflammation is related to an increased risk of tumor formation in many organs, including the liver. Most cases of HCC develop in association with preexisting chronic liver disease and are usually due to hepatitis B virus (HBV), hepatitis C virus (HCV), obesity, or alcohol-associated inflammation (1). For instance, fatty liver disease, a syndrome mimicking an inflammatory condition (2), commonly encompasses hepatic steatosis and steatohepatitis, which have the inherent propensity to progress toward the development of cirrhosis and HCC (3). It is well known that the process of cancer formation consists of the activation of oncogenes and inactivation of tumor suppressors. Nevertheless, the link between loss of tumor suppressor and chronic inflammation-induced cancer occurrence remains obscure, particularly regarding the details of the interplay between inflammation and liver tumorigenesis.

Polycomb group (PcG) proteins, including EZH2 and SUZ12, are epigenetic chromatin modifiers that are involved in the maintenance of embryonic and adult stem cells (4). PcG proteins form multiple polycomb-repressive complexes (PRC) and can be recruited to repress initiation sites and the polycomb-responsive element of target genes. Thus, these PRCs act through epigenetic modification of the chromatin structure to promote gene desensitization (5). The pRB/E2F pathway regulates EZH2 and SUZ12 gene expression (6). However, the regulation between E2F1 and alterations in SUZ12, EZH2, and CEBPD in liver cancer cells remains unknown.

CEBPD is known to respond to the stimulation of inflammatory factors in chronic inflammation-related diseases (7, 8). In addition, the induction of CEBPD induces growth arrest and apoptosis of cancer cells, suggesting that it could act as a tumor suppressor (9). Activation of tumor suppressors is one of the most important strategies in the development of cancer chemotherapy drugs. Hydroxymethyl dibenzoylmethane (HMDB) has been suggested to serve as a potent anticancer drug (10, 11). The p38 MAPK/CAMP response element-binding protein (CREB) pathway contributes to HMDB-induced CEBPD activation (12). Increased CEBPD participates in the transcription of peroxisome proliferator-activated nuclear receptor 2 (PPAR2), an adipogenic and proapoptotic inducer, and DNA-damage-inducible protein 153 (GADD153, also known as CHOP), a proapoptotic inducer (13, 14). A recent study demonstrated that loss of CEBPD might...
contribute to the initiation of breast cancer (15). Moreover, inactivated CEBPD was observed in HCC and cervical cancer via an epigenetic effect (16). Transcription factor Yin-Yang 1 (YY1) physically interacts with SUZ12 and can act as a mediator to recruit PcG proteins and DNA methyltransferases to participate in CEBPD gene desensitization in cancer cells (16). However, details of the molecular mechanism and clinical relevance of CEBPD inactivation in HCC remain unknown.

**Materials and Methods**

**Materials**

5-AzadC was purchased from Calbiochem. HMDB was purchased from Sigma Aldrich. Assay kits for the detection of plasma levels of creatinine and blood urea nitrogen (BlUN) were purchased from BioSystems S.A. Aspartate transaminase (AST) and alanine transaminase (ALT) assay kits were purchased from Human GmbH.

**Data mining of HCC transcriptome dataset**

From a dataset deposited on GEO (National Center for Biotechnology Information, Bethesda, MD), GSE6764, the raw CEL files from normal liver \( n = 10 \) and very early \( n = 8 \), early \( n = 10 \), advanced \( n = 7 \) and very advanced \( n = 10 \). HCC samples were retrieved and imported into Nexus Expression 3 software to analyze the differential expression of \( \text{EZH2} \) in normal and tumorous liver tissue. Next, we also combined the above-mentioned CEL files with the rest 30 dysplastic hepatocellular lesions from the same dataset to examine the associations between \( \text{E2F1}, \text{EZH2}, \text{SUZ12}, \) and CEBPD transcript expressions.

**Patients and tumor specimens**

The Institutional Review Board of the Chi Mei Medical Center (Taiwan) approved the procurement of 27 pairs of snap-frozen HCC and nontumorous liver and 90 paraffin-embedded HCC tissue samples from BioBank of Chi Mei Medical Center for this study (IRB100-11-009). All cases had been treated by surgery with curative intent and were histologically confirmed according to the latest World Health Organization (WHO) classification. Clinicopathological data were obtained from BioBank, including patient gender, age, serum \( \alpha \)-fetoprotein, hepatitis B surface antigen, HCV antibody, the preoperative hepatic function as determined by the Child-Pugh classification, tumor multiplicity, histologic differentiation based on Edmonson and Steiner’s criteria, tumor staging according to the 7th Ed. American Joint Committee on Cancer (AJCC) system, as well as Okuda staging and the staging system from the Cancer of the Liver Italian Program (CLIP). Survival analyses were performed for the 90 cases with paraffin-embedded HCC tissue samples, in which the median period was 40 months (range 1 to 101 months).

**qRT-PCR**

Total RNA of snap-frozen HCC and nontumorous liver samples was extracted, quantified, and subjected to reverse-transcription. Using pre-designed TaqMan assay reagents (Applied Biosystems), we measured the mRNA abundance of CEBPD (Hs00270931_s1) with the ABI StepOnePlus System as previously described (17). The fold expression of CEBPD relative to nontumor counterparts was calculated using the comparative \( \Delta \Delta \)Ct method after normalization to \( \text{POLR2A} \) (Hs01108291_m1) as an internal control.

**IHC staining and assessment**

IHC staining was conducted as described previously (18). The slides were subsequently incubated with a primary antibody targeting E2F1 (1:100), EZH2 (1:50), SLI2 (1:100), or CEBPD (1:100) for 1 hour. We detected primary antibodies using the DAKO ChemMate EnVision Kit. Immunoreactivity was evaluated by an expert pathologist (to C.-F. Li) and based on a combination of the percentage and intensity of positively stained tumor cytoplasm to generate the H-score, which was calculated using the following equation: \( \text{H-score} = \sum_i (i + 1) \), where \( i \) is the intensity of stained tumor cells (0 to 3), and \( P_i \) is the percentage of stained tumor cells for each intensity, varying from 0% to 100%. This formula generates a score range from 100 to 400, while 100 equals 100% of tumor cells showing negative results and 400 equals 100% of tumor cells strongly stained (3+).

**Cell culture and treatments**

HepG2 was acquired from ATCC in 2011. Huh7 was obtained from the Riken Resource Center in 2008. The above cell lines were authenticated by the company using genotypes in 2015. Mouse embryonic fibroblasts (MEF) were isolated essentially as described (19) from individual E13.5–E14.5 embryos generated by mating of Cebpd null heterozygous mice (20) and immortalized by E1A. These cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. In the cotreatment group, 5-AzadC was added to cells (60% confluence) for 48 hours and then switched to medium containing HMDB.

**siRNA and shRNA assays**

siRNA pools for E2F1 and scrambled control siRNA were purchased from Ambion. The lentiviral expression vectors plKO.1-shLuciferase and plKO.1-shCEBPD were purchased from the National RNAi Core Facility located at the Institute of Molecular Biology, Academia Sinica. The virus was produced from Phoenix cells cotransfected with the pMD2.G and psPAX2 vectors along with the plKO.1-shLuc or plKO.1-shCEBPD vector.

**Reverse transcription (RT)-PCR**

We isolated total RNA from Huh7 and HepG2 cells transfected with indicated expression vectors and used CEBPD-, CEBPB-, E2F1-, SLI2-., and EZH2-specific primers for analysis. GAPDH primers were used as a control. Primers used are indicated in the Supplementary Table S4.

**Methylation-specific PCR**

After sodium bisulphite (Zymo research) treatment of genomic DNA, DNA was amplified by PCR using primers specific to methylated sequences. The primers used for amplification of methylated and unmethylated promoters of Cebpd were designed by MethPrimer website.

**Reporter and foci formation assays**

Cells were transfected with plasmids using Lipofectamine 2000 according to the manufacturer’s instructions. Luciferase activity was measured in the lysates of the transfectants as described before (16). The total DNA amount for each experiment was matched equally with control empty vectors. Huh7 and E1A-immortalized wild-type (Cebpd\(^{+/+}\)) or Cebpd\(^{-/-}\) MEF cells were transiently transfected with the indicated expression vectors. After 14 to 21 days, experimental cells were stained with 0.05% crystal violet and the colonies were scored for statistical analysis.
Apoptotic cell death analysis using flow cytometry

Typical cell-cycle histograms were analyzed using flow cytometry after exposure with or without 10 μmol/L 5-AzaC for 48 hours and followed by treatment with 100 μmol/L HMDB. Apoptotic cell death of experimental cells was harvested and fixed in 75% ethanol at −20°C for 24 hours. After incubation with RNase A and stained with propidium iodide (PI) for 1 hour, cells were analyzed on a FACScan cytometer.

Animal studies

Female, 6- to 8-week-old NOD/SCID mice were obtained from the Laboratory Animal Center of National Cheng Kung University (Taiwan). Huh7 cells were inoculated subcutaneously into the right flank of the mice. Animals with macroscopic tumors (50–100 mm³) were placed randomly into four groups (n = 4 per group) as follows: (i) control group; (ii) HMDB treatment group; (iii) 5-AzaC treatment group; and (iv) combined HMDB and 5-AzaC treatment group. Treatment was given to all groups intraperitoneally 5 days per week for 3 weeks. For the DEN/high-fat diets (HDF)-induced HCC model, mice were fed HDF (composition of 59%–fat, 15%–protein, 26%–carbohydrates based on caloric content; TestDiet) for 10 weeks before DEN administration (i.p., 80 mg/kg) at 16 weeks of age, until sacrificed. Tumor and nontumor tissue was collected and rapidly frozen for HCC analysis.

Statistical analysis

All analyses were performed using the statistical software GraphPad Prism version 5 for Windows (GraphPad Software). Quantitative data were expressed as the mean ±SEM of at least three independent experiments. Student t test was used to compare between groups. Statistical significance was accepted for P < 0.05 (*), P < 0.01 (**), or P < 0.001 (***)

The details of the materials and methods used in supplementary figures have been described in the Supplementary Materials and Methods.

Results

Loss of CEBPD is associated with HCC progression

Inactivation of CEBPD was observed in HCC specimens (16); however, a detailed understanding of the physiologic relevance of CEBPD following HCC tumorigenesis is lacking. We first clarified the expression pattern of CEBPD in various liver cancer specimens through analysis of the public dataset GSE6764. We identified significant downregulation of CEBPD in HCC compared with nontumor liver parenchyma (log2 = −1.2685, P = 0.0005), and this downregulation was more significant in very advanced tumors (log2 = −1.4267, P = 0.0002; Fig. 1A). We further validated the pairs of snap-frozen nontumor and HCC samples; the CEBPD transcripts showed a significant stepwise decrease from nontumor tissue to HCC with low and high primary tumor (pT) statuses, respectively (Fig. 1B and D).

As determined by the H-score, lower CEBPD immunoreactivity was significantly associated with numerous adverse clinical features, including multiplicity and poor histologic differentiation, as well as higher pT status, American Joint Committee on Cancer (AJCC) stage and the Cancer of the Liver Italian Program (CLIP) score, suggesting a potential link between CEBPD loss, and HCC progression (Supplementary Table S1). Importantly, combined with numerous important clinicopathologic variables, such as tumor multiplicity, higher pT status, AJCC stage, and a higher CLIP score, the loss of CEBPD expression significantly predicted a worse overall survival (OS; P = 0.0080) and local recurrence-free survival (LRFS; P < 0.0001; Fig. 1E; Supplementary Table S2). Furthermore, the loss of CEBPD expression remained significantly prognostic in a multivariate analysis for LRFS (Supplementary Table S3).

CEBPD is activated in inflammatory hepatocytes and its inactivation is associated with the occurrence of HCC

A number of studies have observed that dietary or genetic obesity are direct promoters of HCC development (21). However, it is not feasible to obtain disease-free human liver specimens. It is known that many regulatory motifs of transcription factors are conserved between the promoters of human CEBPD (CEBPB) and mouse CEBPD (Cebp) genes (22), thus, we examined Cebp abundance in several mouse liver cancer models.

In the livers of HBx Tg mice, inflammation was induced at 8 months and developed HCC at 14 to 18 months (23). Histopathology examination showed that Cebp expression is higher at the inflammatory stage (III, the 12th month) but attenuated at HCC development (IV, the 16th month; Fig. 2A, top). In gene expression profile at different stages of HCC through analysis of the public dataset GSE15251, we found that E2f1 transcripts was consistently high from 12 months (phase of fatty liver) and was sustained in both normal and tumor samples (compared with the 6th-week and 8th-month samples). These results implied that an increase in E2f1 could be the initiator for promoting Suz12 and Ezh2 levels. We further assessed the Cebp transcripts in HBx Tg mice. The result showed that Cebp transcripts were high in inflamed livers (III, the 12th month) but attenuated in liver tumors (IV, the 16th month; Fig. 2A, bottom). Moreover, in a methylation-specific PCR (MSP) assay, compared with nontumor livers (18 months), methylation on the Cebp promoter was increased in the tumors of HBx Tg mice (Fig. 2B). These results were consistent with those obtained in our previous study suggesting that the Cebp promoter is hypermethylated in liver cancer (16). Next, we assessed the expression and role of CEBPD in a HFD/DEN-induced mouse liver cancer model (21). Compared with Cebp-deficient mice, attenuated occurrence of spontaneous liver tumor was observed in Cebp−/− mice (Fig. 2C). These above results support the hypothesis that Cebp plays a tumor suppressor role in liver cells for HCC formation.

E2F1 represses CEBPD transcription in liver cancer cells

The regulation between E2F1 and alterations in SUZ12, EZH2, DNMTs, and CEBPD in liver cancer cells remains uncertain. Exogenous overexpression of HA/E2F1 induced SUZ12 and EZH2 expression and decreased CEBPD expression but had no effect on the expression of DNMTs and CEBPB, another C/EBP family member (Fig. 3A and B). Moreover, the opposing regulation was observed via the knockdown of HA/E2F1 (Fig. 3A and B). From GSE6764, we learned that the abundance of CEBPD transcripts was negatively associated with the levels of E2F1, EZH2, and SUZ12 transcripts (Fig. 3C). Furthermore, the negative associations between CEBPD transcripts and the levels of E2F1, EZH2, and SUZ12 protein expression were further confirmed in 27 snap-frozen HCC samples (Fig. 3D). Taken together, these results suggested that E2F1 is an upstream regulator to induce SUZ12 and EZH2 and deplete CEBPD in HCC.

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Loss of CEBPD enhances E2F1-induced proliferation of liver cancer cells

Consistent with our previous study (16), a reporter assay showed that overexpression of E2F1 could attenuate TNF-α or IL-1β-induced CEBPD reporter activity (Fig. 4A). Next, to assess the effect of CEBPD inactivation on E2F1- or SUZ12-induced cell proliferation, a foci assay was performed using E1A-immortalized Cebpd+/+/ and Cebpd−/− MEFs transfected with an E2F1 or SUZ12 expression vector. The result showed that the increases in E2F1 and SUZ12 significantly induced foci formation in Cebpd−/− MEF cells but did so only slightly in Cebpd+/+ MEF cells (Fig. 4B), suggesting the loss of Cebpd enhances the E2F1 and SUZ12-induced cell proliferation. Furthermore, we assessed the effect of CEBPD existence on E2F1-induced proliferation in liver cancer cells by cotransfection of the HA/E2F1 and CMV promoter-driven CEBPD expression vector (escaping the control of E2F1-induced epigenetic regulation). The result showed that cells expressing exogenous CEBPD attenuated E2F1-induced proliferation activity (Fig. 4C). These findings suggest that activation of CEBPD transcription is affected by E2F1-induced epigenetic regulators SUZ12 and EZH2 and indicate that the loss of CEBPD presents an advantage for the E2F1-induced proliferation of cancer cells.

Figure 1.
Evaluation of the clinical significance of CEBPD expression in hepatocellular carcinoma. A, data mining of transcriptome data (GSE6764) from the public domain demonstrated that CEBPD expression is significantly downregulated in HCC and its attenuation is more significant in very advanced tumors. Confirmation of the downregulation of CEBPD in 27 pairs of HCC specimens in a stepwise manner (B) correlates with tumor stage (C). D, representative IHC assay in two paired HCC specimens showing CEBPD staining. E, both the overall survival and local recurrence-free survival of HCC patients were determined using the Kaplan–Meier method.
Figure 2. CEBPD is activated in inflamed liver and its inactivation is associated with the occurrence of HCC. A, loss of CEBPD was associated with HCC formation in HBx Tg mice. An IHC assay was performed using CEBPD antibody (top). Quantitative data of E2f1, Suz12, Ezh2, and Cebpd transcripts were extracted from the transcript microarray of the livers of HBx Tg and age-matched wild-type mice with SD (bottom). B, sequence locations between −1,600 and +1,000 bp of the Cebpd gene were predicted by the EBI website CpGPlot program. The correlated positions are represented in the scheme. The CpG methylation status of CEBPD promoters in 18-month-old wild-type (WT) or HBx Tg mice was performed using MSP analysis. A visible PCR product in lanes M and lanes U indicates the presence of methylated alleles and unmethylated alleles, respectively. C, wild-type (Cebpd+/−) and Cebpd-deficient B6 mice were kept on normal chow until 6 weeks of age and then were maintained on HFD until 16 months of age. At the 16th week of age, the mice were given DEN until 16 months of age. Mice were sacrificed and analyzed by IHC staining. The IHC images were then quantified in the bottom panel.
CEBPD expression is responsive to HMDB and participates in HMDB-induced apoptosis in liver cancer cells

Our previous studies showed that SUZ12-mediated epigenetic regulation and the p38/CREB pathway could negatively and positively regulate CEBPD expression in cervical cancer, respectively (16, 22). CEBPD is also activated in many differentiation processes and stresses (24, 25). p38 MAPK has been suggested to play a critical role in differentiation and stresses (26, 27). Thus, we
assessed whether CEBPD is responsive to anticancer drugs that are known to activate p38 MAPK, including cisplatin (28), paclitaxel (29), 5-fluorouracil (30), bortezomib (31), and dexamethasone (32) and those that affect epigenetic regulation, such as 5-Aza-C (33) or induce differentiation, such as retinoic acid (34). Western blotting analyses showed that they all induce CEBPD expression (Supplementary Fig. S1). These findings indicate that CEBPD may be a common effector in response to inducers of stresses, epigenetic change, and differentiation. HMDB can induce CEBPD expression via activation of the p38 MAPK/CREB pathway (12). To efficiently activate CEBPD to kill cancer cells, we assessed whether CEBPD expression was higher during the combination of two regulations, activation of the p38/CREB pathway and demethylation of the CEBPD promoter (Supplementary Fig. S2). Consistent with our previous report in cervical cancer (12), a reporter assay showed that the dominant negative form of p38 and CREB repressed HMDB-induced CEBPD reporter activity in liver cancer cells (Supplementary Fig. S3A). PI staining revealed that CEBPD contributed to HMDB-induced apoptosis of liver cancer cells (Supplementary Fig. S3B).

CEBPD can induce PPAR2 and GADD153 expression to promote apoptotic cell death (12). CEBPD, PPAR2, and GADD153 were coordinately activated by HMDB or 5-Aza-C (Fig. 5A). Compared with individual treatments, combinatorial treatment of HMDB and 5-Aza-C additively increased the expression of CEBPD and its downstream targets (Fig. 5B). The effects of liver cancer cells in responsive to HMDB, 5-Aza-C or combinatorial treatment of HMDB and 5-Aza-C were assessed by examining the sub-G1 population, caspase3/8 activity, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Consistent with higher CEBPD expression due to combinatorial treatment of HMDB and 5-Aza-C, the sub-G1 population of liver cancer cells was higher when the cells were treated with combined HMDB and 5-Aza-C (Fig. 5C and Supplementary Fig. S4). Furthermore, the active forms of caspase-3 and caspase-8 in response to the combinatorial treatment of HMDB and 5-Aza-C were greater than the individual treatment of HMDB or 5-Aza-C (Supplementary Fig. S5A). In addition, the result of TUNEL assay demonstrated a coincident manner as mentioned above (Supplementary Fig. S5B). These data suggested that administration of these two drugs together induced an additive effect. Next, we investigated whether CEBPD was involved in dual drug-induced cell death. These result showed that the loss of CEBPD attenuated dual drug-induced death of liver cancer cells (Fig. 5D and Supplementary Fig. S6). These results suggested that higher CEBPD levels were also associated with higher apoptotic activity in cancer cells.

**CEBPD is required for the antitumor activity of HMDB and 5-Aza-C combinatorial treatment in a Huh7 xenograft tumor model**

We further evaluated the combined effect by comparing with the effect of single treatments in human tumor xenograft mice. In addition, considering the severe side effects of high-dose 5-Aza-C when it is administered in clinical therapy (35, 36), we used a relatively lower dose of 5-Aza-C combined with HMDB to treat Huh7 cell xenograft in NOD-SCID mice. In Huh7 xenografts, treatment with HMDB or 5-Aza-C inhibited tumor growth compared with control. Moreover, the combinatorial treatment of HMDB and 5-Aza-C produced a significantly enhanced antitumor activity compared with single-agent treatments (Fig. 6A).

Importantly, mice tolerated the treatment of both HMDB and 5-Aza-C well, as no body weight losses or toxicity-related deaths were observed during or after treatment (Fig. 6B and 6C). In addition, loss of CEBPD attenuated the dual treatment-enhanced killing effect in Huh7 cell xenografted NOD-SCID mice (Fig. 6D), suggesting that CEBPD activation is important for the antitumor activity of 5-Aza-C and HMDB.

**Discussion**

In this study, we provide the first evidence that CEBPD loss encourages liver cancer occurrence and exhibits a significant association with the outcomes for HCC patients. In liver cells,
Figure 5.
Effects of combinatorial treatment of HMDB and 5-AzadC in liver cancer cells. A, Huh7 and HepG2 cells were incubated with 5-AzadC at the indicated doses for 48 hours or HMDB (60 µmol/L) at the indicated time points. Total lysates were harvested for Western blotting analysis. B, Huh7 and HepG2 cells were treated with 5-AzadC for 48 hours and then incubated with HMDB at the indicated concentration. Total lysates were harvested for Western blotting analyses. C, typical cell-cycle histograms were analyzed using flow cytometry after exposure in the presence or absence of 5-AzadC for 48 hours, followed by HMDB at the indicated time points. D, Huh7 and HepG2 cells were infected with lentiviruses encoding shLacZ or shCEBPβ and subsequently treated with or without HMDB and 5-AzadC; the cells were then harvested for typical cell-cycle histograms. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
CEBPD is responsive to the proinflammatory cytokines, IL1β and TNFα. The physiologic relevance of higher levels of CEBPD was observed in human clinical specimens and in nontumor samples from HBx Tg mice. However, CEBPD expression is attenuated in HCC specimens and in liver tumors of HBx Tg mice. Moreover, our results suggested that inactivation of CEBPD is regulated by

![Graph showing tumor growth and body weight over time for different treatment groups.](image)

**Figure 6.** Intraperitoneal combined treatment of HMDB and 5-AzadC suppresses the growth of Huh7-xenograft in NOD-SCID mice. Huh7 cells were subcutaneously inoculated into NOD-SCID mice, followed by an intraperitoneal injection of vehicle, 5-AzadC (0.1 mg/kg/d), HMDB (50 mg/kg/d), or HMDB (50 mg/kg/d) combined with 5-AzadC (0.1 mg/kg/d). A and B, tumor dimensions and animal weights were obtained at the indicated time points. C, following 3-week drug treatment, all of the animals were sacrificed, and their plasma was collected. Plasma levels of the liver-specific enzymes ALT, AST, and renal function tests, such as BUN and creatinine levels, were measured. D, Huh7-shLacZ (shLacZ) and Huh7-shCEBPD (shD) cells were subcutaneously inoculated on the dorsal of 6-week-old NOD-SCID mice (n = 4) followed by intraperitoneal injection of HMDB combined with 5-AzadC. *** P < 0.001; N.S., not significant.
E2F1, SUZ12, and EZH2 expression, which may coordinate to promote liver cancer progression. We further demonstrated that CEBPD could be reactivated in liver cancer cells upon treatment with clinically used anticancer drugs, including HMDB and 5-AzadC. Following combinatorial treatment of HMDB and 5-AzadC, additive effects were observed in the expression of CEBPD and its downstream proapoptotic genes; this observation was consistent with findings of the enhancement of death of cancer cells. Furthermore, an anticancer effect was consistently observed in the xenograft cancer formation assay.

Chronic inflammation results in increased production of reactive oxygen species and a decrease in cell-mediated immunity. Infections caused by HBV and HCV affected the progression of cancer because these infections cause cancer when chronic inflammation occurs (37). The inflammatory factors NFKB and STAT3 were sustained and play crucial roles in inducing cancer formation (38). Here, we demonstrated that inactivation of another inflammation-responsive factor, CEBPD, by the epigenetic E2F1/SUZ12/EZH2 axis contributes to liver tumorigenesis. Importantly, inactivation of CEBPD during cancer progression has been reported in breast cancer, cervical cancer, HCC, leukemia, and prostate cancer (16, 39, 40). In addition to the proapoptotic genes PPGAR2 and GADD153 (41, 42), our recent results suggested that CEBPD contributes to the transcriptional activation of procaspase-8 in prostate cancer (43). These observations further support the hypothesis that activation of CEBPD contributes to the induction of death signaling in cancer cells.

CEBPD participates in the induction of genomic instability inducers, including aurora kinase C, superoxide dismutase 1 (SOD1), and COX-2 (28, 44). Genomic instability has been suggested to link to anticancer drug resistance and cancer recurrence (44). The increase in genomic instability may contribute to chemotherapeutic resistance to cancer therapy, such as cisplatin (45). In this study, we showed that several clinically administered drugs that induce cellular stress (paclitaxel, 5-fluorouracil, and actinomycin D) or differentiation (dexamethasone and retinoic acid) can induce CEBPD expression (Supplementary Fig. S1). However, hypermethylation of the CEBPD promoter could attenuate the sensitivity of CEBPD in response to anticancer drugs. These observations suggest that CEBPD is desensitized in response to anticancer drugs due to hypermethylation of the CEBPD promoter in cancer cells, which may easily develop to drug-resistant cancer. Thus, stronger activation of CEBPD, as shown here, could be therapeutically relevant in cancer treatment.

We propose that the combinatorial treatment of HMDB and 5-AzadC could be useful for cancer therapy. However, many combinations of anticancer drugs can be tested with the goal of stronger activation of CEBPD in cancer cells. For example, in addition to DNA hypermethylation of the CEBPD promoter region, the lysine 27 residue on histone 3 is methylated in the same area. In addition to inhibitors targeting DNA methylation, several anticancer drugs that are in the clinical trial phase, such as 3-deazaadenosine (DZA) and neplanocin A (Nep A) (46), have been developed to target polycomb proteins SUZ12 and EZH2. In the future, we propose to combine these therapies with CEBPD inducers, such as cisplatin, paclitaxel, 5-fluorouracil, and bortezomib, particularly in cancer with CEBPD desensitized by the E2F1-, SUZ12/EZH2- or DNMTs-mediated epigenetic effect. In this study, we found that inactivation of CEBPD by the E2F1-regulated epigenetic axis contributes to liver tumorigenesis. In addition, we successfully confirmed that combined treatment of HMDB and low-dose 5-AzadC can reactivate CEBPD expression and strengthen apoptosis effects in HCC. Taken together, these results suggest that CEBPD could be a useful diagnostic marker and therapeutic target in HCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: C.-F. Li, J.-M. Wang

Development of methodology: C.-F. Li

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.-H. Tsai, C.-Y. Ko, C.-J. Yen, H.-Y. Lai, W.-C. Wu

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.-M. Wang, H.-H. Tsai, Y.-C. Pan, H.-Y. Lai, W.-C. Wu

Writing, review, and/or revision of the manuscript: C.-F. Li, J.-M. Wang, H.-H. Tsai, H.-Y. Lai

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.-F. Li

Other (checked and provided the expression patterns of CCAAT/enhancer-binding protein delta, SUZ12, EZH2 and E2F1 in using the microarray data from HBx transgenic mice): C.-H. Yuh

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