HDAC1-Dependent Repression of Markers of Hepatocytes and P21 Is Involved in Development of Pediatric Liver Cancer

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SUMMARY
Dedifferentiation of hepatocytes into cancer cells is a first step in the development of liver cancer. In this article, we show that the dedifferentiation of hepatocytes involves repression of markers of hepatocytes and the increase of liver proliferation, which are mediated by histone deacetylase 1-dependent repression of promoters of markers of hepatocytes and p21.

BACKGROUND & AIMS: Epigenetic regulation of gene expression plays a critical role in the development of liver cancer; however, the molecular mechanisms of epigenetic-driven liver cancers are not well understood. The aims of this study were to examine molecular mechanisms that cause the dedifferentiation of hepatocytes into cancer cells in aggressive hepatoblastoma and test if the inhibition of these mechanisms inhibits tumor growth.

METHODS: We have analyzed CCAAT/Enhancer Binding Proteins alpha (C/EBPα), Transcription factor Sp5, and histone deacetylase (HDAC)1 pathways from a large biobank of fresh hepatoblastoma (HBL) samples using high-pressure liquid chromatography–based examination of protein–protein complexes and have examined chromatin remodeling on the promoters of markers of hepatocytes and p21. The HDAC1 activity was inhibited in patient-derived xenograft models of HBL and in cultured hepatoblastoma cells and expression of HDAC1-dependent markers of hepatocytes was examined.

RESULTS: Analyses of a biobank showed that a significant portion of HBL patients have increased levels of an oncogenic de-phosphorylated-S190-C/EBPα, Sp5, and HDAC1 compared with amounts of these proteins in adjacent regions. We found that the oncogenic de-phosphorylated-S190-C/EBPα is created in aggressive HBL by protein phosphatase 2A, which is increased within the nucleus and dephosphorylates C/EBPα at Ser190. C/EBPα–HDAC1 and Sp5–HDAC1 complexes are abundant in hepatocytes, which dedifferentiate into cancer cells. Studies in HBL cells have shown that C/EBPα–HDAC1 and Sp5–HDAC1 complexes reduce markers of hepatocytes and p21 via repression of their promoters. Pharmacologic inhibition of C/EBPα–HDAC1 and Sp5–HDAC1 complexes by Suberoylanilide hydroxamic acid (SAHA) and small interfering RNA–mediated inhibition of HDAC1 increase expression of hepatocyte markers, p21, and inhibit proliferation of cancer cells.

CONCLUSIONS: HDAC1-mediated repression of markers of hepatocytes is an essential step for the development of...
Liver cancer is the fifth most common cancer and the third most common cause of cancer-related death in the world.\(^1\) It recently was established that the cellular origin of hepatocellular carcinoma (HCC) and hepatoblastoma (HBL) are hepatocytes that undergo dedifferentiation into cancer cells.\(^2-6\) A portion of HBL patients have an aggressive form of HBL that is characterized by metastases, multiple nodules at diagnosis, vascular invasion, chemoresistance, and relapse.\(^7-9\) Recent publications have shown that epigenetic silencing by histone deacetylases (HDACs) might be critical for the development of aggressive pediatric liver cancer.\(^10\) In particular, an increase of HDAC1 and HDAC2 proteins was found in a large group of patients with HBL.\(^11\) One of the key transcription factors that bring HDAC proteins to the promoters is CCAAT/Enhancer Binding Protein alpha (C/EBP\(\alpha\)). C/EBP\(\alpha\) is a tumor-suppressor protein that arrests cell proliferation by inhibiting cyclin dependent kinases 2 and 4 (cdk2/4) and Transcription factor that binds to E2 recognition site (E2F) signaling.\(^9,12\) However, recent studies have shown that C/EBP\(\alpha\) is increased in a group of patients with liver cancers and shows oncogenic activities.\(^5,6,13,14\) The oncogenic activities of C/EBP\(\alpha\) are associated with mutations or post-translational modifications occurring in the region of C/EBP\(\alpha\) between amino acids 180 and 200.\(^6,15\) This 20-amino acid region is rich for prolines and contains Ser190. Our studies have shown that, in adult cancers and in cancer cells, C/EBP\(\alpha\) is dephosphorylated by protein phosphatase 2A (PP2A) at Ser190 and loses its growth-inhibitory activities.\(^11,12\) Recent studies of pediatric liver cancer identified a group of patients with aggressive HBL who have high levels of C/EBP\(\alpha\), dephosphorylated at Ser190.\(^5,6\) Normally, phosphorylated phospho-S190-C/EBP\(\alpha\) activates transcription of liver-specific genes via interactions with chromatin remodeling protein p300.\(^16,17\) However, the de-phosphorylated-S190 C/EBP\(\alpha\) does not interact with p300 and has reduced ability to activate target genes.\(^17\)

In addition to C/EBP proteins, Sp family proteins also are involved in the regulation of biological processes in the liver including liver proliferation and cancer.\(^10\) A critical target of the Sp family is cdk inhibitor p21. It has been shown that the promoter of p21 contains 6 Sp binding sites in very close proximity to the start of transcription and that Sp1/3 up-regulates the expression of p21.\(^19\) However, if Sp1/3 are associated with HDAC1, they repress p21 promoter.\(^20,21\) It also has been shown that inhibition of HDAC releases this repression.\(^20,22\) In this article, we present evidence that another member of the Sp family, Sp5, is increased in patients with aggressive HBL, forms complexes with HDAC1, and down-regulates p21 by repression of the p21 promoter.

**Results**

**PP2A-Oncogenic C/EBP\(\alpha\) Pathway Is Activated in Patients With Aggressive HBL**

It has been shown that the oncogenic C/EBP\(\alpha\) and Sp5 are increased in patients with HBL.\(^5,6,13,14\) Because both proteins interact with HDAC1, we first examined their levels in our fresh biobank (22 HBL samples obtained immediately after surgery). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis showed that approximately 30% of the fresh biobank contains HBL samples with an increase of both C/EBP\(\alpha\) and HDAC1. A high increase of Sp5 was observed in a larger portion of the fresh samples, including all samples with increased C/EBP\(\alpha\) and HDAC1. In Patients With Aggressive HBL (Figure 1A). The growth-inhibitory region of C/EBP\(\alpha\) is located between amino acids 180 and 200. We found that the oncogenic form of C/EBP\(\alpha\) is dephosphorylated at Ser190, which is located in a growth-inhibitory region and is surrounded by 12 prolines, 7 of which are located upstream of Ser190 and 5 are located downstream of Ser190 (Figure 1B). Mutations of Ser190 and amino acids of the surrounding region have been found previously in patients with HCC. Our studies of HCC and HBL have shown that C/EBP\(\alpha\) is dephosphorylated at Ser190 in these cancers.\(^5,6,14,15\)

Therefore, we asked if C/EBP\(\alpha\) is dephosphorylated at Ser190 in a fresh biobank. For these studies, we initially selected 4 HBL samples with the highest increase of C/EBP\(\alpha\) and HDAC1 (Figure 1A). Examination of levels of total C/EBP\(\alpha\) protein and levels of phospho-S190-C/EBP\(\alpha\) in these fresh HBL samples found that total C/EBP\(\alpha\) is increased in HBL patients, but the phospho-S90 isoform is reduced dramatically compared with background regions (Figure 1C). This result shows that the increased C/EBP\(\alpha\) is dephosphorylated at Ser190. Because previous reports have shown that PP2A dephosphorylates C/EBP\(\alpha\) at Ser190 in cancers,\(^5,6,14,15\) we examined levels of a catalytic subunit of PP2Ac and found that it is increased in nuclear extracts of aggressive HBL, but it is reduced in cytoplasmic fractions of HBL. Examination of whole-cell extracts showed identical levels of PP2Ac in background and tumor regions of HBL patients, suggesting translocation of PP2A from cytoplasm to nucleus. Examination of HDAC1 showed that this protein also is increased in nuclear extracts from tumors of HBL patients. We next examined levels of the oncogene Gank, which degrades the

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**Abbreviations used in this paper:** ALCD, aggressive liver cancer domain; C/EBP\(\alpha\), CCAAT/Enhancer Binding Protein alpha; cdk2/4, cyclin dependent kinases 2 and 4; ChIP, chromatin immunoprecipitation; Co-IP, co-immunoprecipitation; HBL, hepatoblastoma; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; HNF, Hepatocyte Nuclear Factor; mRNA, messenger RNA; MW, molecular weight; NRF2, Nuclear Factor erythroid-2; OCT, organic cation transporter; Ola, Olaparib; PARP, Poly [ADP-ribose] polymerase; PDX, patient-derived xenograft; PP2A, protein phosphatase 2A; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; SEC, size exclusion chromatography; SLC, solute carrier.

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phosphorylated ph-S190-C/EBPα isoform and observed an increase of Gank in these aggressive HBL samples (Figure 1C). However, next co-immunoprecipitation (Co-IP) studies found that Gank does not interact with the de-phosphorylated-S190-C/EBPα, which explains why oncogenic C/EBPα is not degraded in HBL. Further Co-IP studies have shown that de-phosphorylated-S190-C/EBPα interacts with PP2Ac and HDAC1 (Figure 1D). We next examined if the increase of PP2Ac in the nucleus might be associated with translocation of PP2Ac from the cytoplasm to the nucleus. PP2A consists of 3 subunits: scaffold subunit A, catalytic subunit C, and regulatory subunits B.24 The subunit B consists of several molecules with different functions.25 Among these subunits, Protein phosphatase 2, regulatory subunit B (B56), alpha isoform (PPP2R5A) is increased in HCC and shuttles between the cytoplasm and nucleus, determining the intracellular location of the catalytic PP2Ac subunit.26 Therefore, we asked if the PPP2R5A might translocate catalytic subunit PP2Ac to the nucleus in aggressive HBL patients. Western blot showed that, in background regions, PPP2R5A is detectable as a single band (isoform) and that this isoform is increased in tumor sections. Most importantly, in tumor sections, we detected the appearance of slowly migrating immunoreactive PPP2R5A in nuclear extracts of livers of wild-type (WT) and C/EBPα-S193A mice, and HDAC1 was examined in these IPs. Two to 3 technical replicates were performed for each biological sample.

**Figure 1. Oncogenic C/EBPα–HDAC1 pathway is activated in patients with aggressive HBL.** (A) Levels of HDAC1, C/EBPα, and Sp5 mRNAs in fresh biobank HBL samples. Quantitation was performed using 22 HBL tumor samples and 22 corresponding background (adjacent) regions. P values are as follows: HDAC1 (***P < .001), C/EBPα (*P < .05), and Sp5 (**P < .01). (B) Cancer-related mutations and modifications in the growth-inhibitory region of C/EBPα. (C) Western blot showing expression of proteins in tumor sections and in background regions of patients with HBL. Representative results are shown for 4 HBL tumor sections and 3 background regions. Two to 3 technical replicates were performed for each biological sample. (D) Co-IP studies. Upper: IP with antibodies to total C/EBPα and Western blot with antibodies to PP2Ac, gankyrin, and HDAC1. Bottom: IP of HDAC1 and Western blot with antibodies to ph-S190-C/EBPα and to total C/EBPα. Two repeats with 4 samples were performed. (E) PPP2R5A interacts with catalytic subunit PP2Ac in nuclei of HBL patients. Upper: Western blot for proteins shown on the right. Red arrows show slow-migrating isofrom of RRR2R5A, black arrows shows fast-migrating isofrom. Middle: Co-IP; bottom: Western blot shows cell-cycle proteins. Two to 3 technical replicates were performed for each biological sample. (F) Upper: Western blot for C/EBPα and HDAC1. Bottom: Co-IP studies. C/EBPα was immunoprecipitated from nuclear extracts of livers of wild-type (WT) and C/EBPα-S193A mice, and HDAC1 was examined in these IPs. Two to 3 technical replicates were performed for each biological sample.
type C/EBP\(\alpha\) has a weak interaction with HDAC1; however, the mutant C/EBP\(\alpha\)-S193A strongly interacts with HDAC1 (Figure 1F). Because the mutant C/EBP\(\alpha\)-S193A cannot be phosphorylated at Ser193, these studies showed that the dephosphorylation of C/EBP\(\alpha\) at Ser190/193 is critical for strong interactions with HDAC1. The increased interactions of the mutant C/EBP\(\alpha\)-S193A with HDAC1 in this animal model also suggest that the mutations of human C/EBP\(\alpha\) at Ser190 observed in HCC (Figure 1B) create a protein that might interact with HDAC1 in human liver cancers and possesses oncogenic activities.

**HDAC1 and Oncogenic Form of C/EBP\(\alpha\) Are Observed in Hepatocytes That Dedifferentiate to Cancer Cells**

Because de-phosphorylated-S190-C/EBP\(\alpha\) creates dedifferentiating hepatocytes with intranuclear inclusions, we asked if the increased HDAC1 and C/EBP\(\alpha\) are located in the dedifferentiating hepatocytes. Immunohistochemistry of 3 additional fresh HBL samples showed that HDAC1 is located in dedifferentiating hepatocytes that are a large size and have intranuclear inclusions (Figure 2A). Similar to HDAC1, C/EBP\(\alpha\) also is abundant in nuclei of large dedifferentiating hepatocytes and in hepatocytes with mitotic figures (Figure 2B). Staining with antibodies to marker of hepatocytes Hepatocyte Nuclear Factor 4 Alpha, HNF4\(\alpha\) confirmed that the large cells with intranuclear inclusions are hepatocytes (Figure 2C). Western blot with antibodies to total C/EBP\(\alpha\) and to ph-S190-C/EBP\(\alpha\) showed that de-phosphorylated-S190-C/EBP\(\alpha\) is increased in these HBL patients (Figure 2D). HDAC1 protein also is increased in these HBLs. Co-IP studies and size exclusion chromatography (SEC) showed that the oncogenic C/EBP\(\alpha\) and HDAC1 form complexes within these fresh HBL samples (Figure 2D and E). Thus, immunostaining and molecular investigations of HBL patients showed that HDAC1 and C/EBP\(\alpha\) are located in dedifferentiating hepatocytes and form complexes. The case of HBL3 is a rare case, and is described later.

**Identification of the Rare HBL Patient Who Has Activated HDAC1, C/EBP\(\alpha\), and Sp5, While Other Pathways of Liver Cancer Are Reduced**

After resections, we performed characterization of HBL samples by qRT-PCR–based analyses of stem cell markers,
markers of hepatocytes, and major cancer pathways of HBL including β-catenin, Nuclear factor erythroid-2 (NRF2), and Gank. Figure 2F shows such qRT-PCR analysis of the HBL sample, which has an expression profile identical to those in other HBL samples, and analysis of the rare HBL3 sample, which has significant differences. Stem cell markers and oncogenic pathways, including β-catenin, NRF2, Gank, oncogenic C/EBPα, HDAC1, and Sp5, are increased in representative HBL samples. However, the rare sample HBL3 has a reduction of β-catenin, NRF2, and Gank, along with an increase of HDAC1-C/EBPα and HDAC1-Sp5 pathways (Figure 2F). This increase of the C/EBPα-HDAC1 pathway in this patient on protein levels is shown in Figure 2D and E. This suggests that the activation of HDAC1-dependent chromatin silencing is critical and, in some cases, might be the major contributor for the development of HBL.

Sp5 Forms Complexes With HDAC1 in HBL Patients

Given the increase of Sp5 messenger RNA (mRNA) in patients with HBL, we next examined protein levels of Sp5. Western blot analysis of 8 HBL samples found that protein levels of Sp5 are 2.5- to 3.5-fold increased in patients with HBL (Figure 3A). Examination of a target of Sp5-HDAC1 p21 showed that p21 is reduced (Figure 3A). Co-IP studies showed that Sp5 and HDAC1 form complexes in aggressive HBL (Figure 3B). We next asked if the Sp5–HDAC1 complexes were observed in our rare HBL3 case shown in Figure 2F. SEC showed that both HDAC1 and Sp5 are located in high molecular weight (MW) regions of SEC and form a complex (Figure 3C). To examine if HDAC1–Sp5 complexes repress the p21 promoter, we performed a chromatin immunoprecipitation (ChIP) assay. Because the p21 promoter contains 6 SP binding sites in close proximity to the start of
transcription\(^{19}\) (Figure 3D), we used PCR primers that cover this region of the promoter. Figure 3D shows that, in background regions, HDAC1 and Sp5 are not bound to the p21 promoter. However, HDAC1 and Sp5 occupy the p21 promoter in HBL tumors, which correlates with methylation of H3K9, reflecting repression of the promoter. Because HDAC1 does not bind directly to DNA, these results suggest that Sp5 delivers HDAC1 to the p21 promoter. Taken together, these studies show that the Sp5–HDAC1 axis is activated in aggressive HBL and is involved in the repression of p21 (Figure 3E).

**De-phosphorylated-S190-C/EBP\(\alpha\)–HDAC1 Complex Occupies Promoters of PECK and CYP3A4 Genes and Represses Their Expression in HBL Patients**

Our previous examination of a large cohort of HBL patients showed that a family of cytochrome genes (CYP), markers of hepatocytes, are reduced dramatically.\(^4\) Therefore, we searched promoter regions of the CYP family of genes with higher levels of reduction in HBL and found that all of them contain 2–4 C/EBP\(\alpha\) binding sites (Figure 4A), suggesting that they might be targets of C/EBP\(\alpha\)–HDAC1 complexes. qRT-PCR analysis of a fresh biobank showed that mRNAs of CYP3A4 and PECK (another marker of hepatocytes) are reduced in the fresh biobank of HBL samples, while markers of stem cells Thy1 and Oct4 are increased (Figure 4F). Therefore, we looked at the levels of the PECK, CYP3A4, and stem cell marker proteins and found that PECK, CYP3A4, CYP7A1, and CYP2B6 also are reduced in tumor sections of fresh aggressive HBL, while Thy1 and Oct 4 are increased (Figure 4C). ChIP assay showed that the promoters of PECK and CYP3A4 genes are activated in background regions by C/EBP\(\alpha\)–p300 complexes; however, these promoters are repressed in tumor sections by C/EBP\(\alpha\)–HDAC1 complexes (Figure 4D and E). Based on these studies of tumor samples from HBL patients, we suggested that, in aggressive HBL, a regulatory PPP2R5A subunit translocates the catalytic subunit PP2Ac to the nucleus, where PP2Ac dephosphorylates C/EBP\(\alpha\) at Ser190, leading to formation of C/EBP\(\alpha\)–HDAC1 complexes. These complexes repress promoters of markers of hepatocytes CYP3A4 and PECK, resulting in reduction of these proteins (Figure 4F). We next confirmed this hypothesis in HBL cells.

**Inhibition of PP2A in Cancer Cells Eliminates de-phosphorylated-S190-C/EBP\(\alpha\)–HDAC1 Complexes**

We first asked if the inhibition of an initial step of the oncogenic PP2Ac–C/EBP\(\alpha\) pathway, PP2A activity, by pharmacologic approach reduces the oncogenic form of C/EBP\(\alpha\)
and levels of C/EBPα–HDAC1 complexes. To inhibit PP2A activity, we used LB100, which is highly specific to PP2A27 (Figure 5A). We found that the treatment of Huh6 cells with 5 μmol/L LB100 for 48 hours restores phosphorylation of C/EBPα at Ser190 (Figure 5B) and eliminates C/EBPα–HDAC1 complexes (Figure 5C). The SEC approach also was used and surprisingly showed a dramatic reduction of the large region of optical density in the region of high MW complexes of SEC (Figure 5D), suggesting that PP2A controls multiple high-MW protein–protein complexes. Examination of C/EBPα and HDAC1 in SEC fractions showed that, in untreated cells, both proteins are located in high-MW fractions and form a complex. The inhibition of PP2A by LB100 destroys the complexes and shifts the proteins to lower MW fractions.

**Pharmacologic Inhibition of PP2A and HDAC1 Inhibits Proliferation of Cancer Cells**

We next examined if Food and Drug Administration–approved inhibitors of HDAC1, SAHA (vorinostat), and LB100 might inhibit proliferation of Huh6 cells. Huh6 cells were treated with LB100, SAHA, or with the combination of LB100 + SAHA. Figure 6A shows that total protein levels of C/EBPα and HDAC1 are increased slightly by LB100 and SAHA correspondingly. In addition, it shows that C/EBPα is phosphorylated at Ser190 in cells treated with LB100, or with the combination of LB100 and SAHA, but not in cells with SAHA alone. Interestingly, Western blot with antibodies to Poly [ADP-ribose] polymerase 1 (PARP1) showed the appearance of cleaved forms of PARP1, indicating apoptosis, mainly in cells treated with a combination of drugs (Figure 6A, red arrow). HDAC1–C/EBPα complexes are eliminated in cells treated with LB100 and with the combination of LB100 + SAHA (Figure 6B). Interestingly, the rescue of phosphorylation of C/EBPα at Ser190 restores its ability to form complexes with p300, which are activators of many C/EBPα-dependent genes.16,17 Our further ChIP studies showed that the LB100-restored C/EBPα–p300 complexes are involved in activation of PEPCK and CYP3A4 (Figure 7D).

Examination of proliferation found that treatments of Huh6 cells with SAHA and LB100 reduce proliferation of the cells, along with combined treatments that lead to further inhibition of proliferation (Figure 6C). We found that untreated Huh6 cells form large clusters containing multiple cells, but treatment with SAHA or LB100 alone reduces the size of the cell clusters. Combined treatments resulted in an even smaller size of clusters (Figure 6D). The terminal deoxynucleotidyl transferase–mediated deoxuryridine triphosphate nick-end labeling assay found that LB100 or SAHA alone initiated apoptosis, but combined treatments lead to a higher degree of cell death (Figure 6D, bottom). Examination of cell-cycle proteins showed that cdk2, cyclin D1, cdc2, and cyclin E were
reduced in cells treated with LB100 or SAHA alone, whereas combined treatments resulted in the strongest inhibition of these proteins (Figure 6E and F); thus showing that the inhibition of PP2A by LB100 destroys C/EBPα–HDAC1 complexes and inhibits proliferation of cancer cells.

Elimination of C/EBPα–HDAC1 Complexes Increases Levels of Markers of Hepatocytes via Removal of C/EBPα–HDAC1 Complexes From the Promoters

In addition to the CYP family, 35 members of another family of proteins, organic cation transporters (OCT or SLC) are reduced in HBL patients.4,5 We analyzed promoters of the 7 members of the OCT family with the highest levels of reduction and found that examined SLC genes contained 1–3 C/EBPα sites in their promoters (Figure 7A). Figure 7B shows that, in hepatoblastoma HepG2 and Huh6 cell lines, the LB100-mediated elimination of C/EBPα–HDAC1 complexes leads to activation of 5 members of the CYP family: CYP2C8, CYP3A4, CYP1A2, CYP2A6, and CYP2B6. We observed a more complicated pattern of expression of OCT genes. The members of OCT family SLC22A7, SLC2A4, and SLC22A18 are increased in cells treated with LB100, whereas SAHA increased expression of SLC22A1, SLC2A7, SLC2A2, and SLC2A4 genes. Examination of protein levels of CYP3A4, CYP2B6, and PEPCK showed that LB100 and SAHA increased the levels of these proteins (Figure 7C). ChIP assay showed that, in untreated HepG2 cells, C/EBPα and HDAC1 occupy and repress PEPCK and CYP3A4 promoters. However, the combined treatments of HepG2 cells with SAHA and LB100 remove these complexes and activate the promoters (Figure 7D). It is interesting that LB100 + SAHA treatments not only remove C/EBPα–HDAC1 complexes from the promoters, but also restore C/EBPα–p300 complexes that occupy the PEPCK and CYP3A4 promoters in treated cells (Figure 7D). This switch of the complexes is similar to the patterns observed in tumor and background sections of the HBL patients (Figure 4D).
Olaparib-Mediated Reduction of HDAC1 and Subsequent Reduction of C/EBPα–HDAC1 Complexes in Patient-Derived Xenografts Increases Expression of Markers of Hepatocytes

We previously showed that the increase of many oncogenes in HBL is mediated by activation of specific regions of the genome in what we refer to as aggressive liver cancer domains (ALCDs), and that PARP1 activates ALCDs in aggressive HBL.5 We recently found that the inhibition of PARP1 by olaparib in patient-derived xenografts (PDXs) reduces levels of the ALCD-dependent oncogene, inhibits tumor growth, and increases expression of hepatocyte markers CYP3A4 and CYP2A6.28 Because these CYP genes are direct targets of C/EBPα–HDAC1 complexes, we suggested that HDAC1 might be reduced in PDXs treated with Olaparib (Ola) by an ALCD-dependent pathway. Therefore, we carefully searched the HDAC1 gene and identified an ALCD within the first intron with 90% homology to other ALCDs. This ALCDs contain the perfect binding site for the ph-S6–p53–PARP1 complexes. ChIP assay showed that the ph-S6–p53–PARP1 complexes occupy and activate the ALCDs in untreated PDXs and that olaparib removes the complexes and represses this locus of the HDAC1 gene (Figure 7E). We next performed Western blot of 6 vehicle-treated and 6 Ola-treated PDXs. Western blot was performed twice with 6 PDXs from 3 HBL patients. Western: Expression of CYP family proteins and p21 in olaparib-treated PDXs. Western blot was performed twice with 6 PDXs from 3 HBL patients. (G) ChIP assay with the CYP3A4 and p21 promoters. The ChIP assay was performed once owing to limited amounts of the tissues. IgG, signals of heavy chains of IgG. HD, HDAC1; LB, LB 100; Un, untreated cells.
removes the complexes from the promoter, leading to the opening of ALCD as H3K9 acetylation shows (Figure 7G).

Taking studies in cultured cells and PDXs together, we conclude that the elimination of C/EBPα–HDAC1 complexes by LB100þSAHA or by Ola activates promoters of PEPCK and CYP3A4, and that reduction of Sp5-HDAC1 activates the p21 promoter.

Inhibition of HDAC1 Activity by SAHA Removes Sp5–HDAC1–Mediated Repression of the p21 Promoter and Increases Levels of p21

Because HDAC1–Sp5 complexes repress p21 in HBL patients (Figure 3D), we examined if inhibition of HDAC1 in cancer cells might release this repression and restore expression of p21. qRT-PCR showed that levels of Sp5 in HBL cells HepG2 and Huh6 are 100–500 times higher than in non-HBL HeLa cells (Figure 8A). Co-IP studies showed that Sp5 forms complexes with HDAC1 in HepG2 and Huh6 cells (Figure 8B). We next asked if inhibition of HDAC1 by SAHA de-represses p21. Western blot showed that p21 is increased in cells treated with SAHA and with the combination of SAHA + LB100 (Figure 8C). The SEC approach showed that, similar to treatments with LB100 alone, the combination SAHA + LB100 leads to a reduction of optical density in the region of high-MW complexes (Figure 8D). Western blot and Co-IPs showed that treatments with LB100 + SAHA destroy the Sp5–HDAC1 complexes. ChIP assay found that treatments of HepG2 and Huh6 cells with LB100 + SAHA remove Sp5–HDAC1 complexes from the p21 promoter (Figure 8E). To examine if HDAC1 is responsible for repression of p21, we inhibited HDAC1 by specific small interfering RNA and found that p21 is increased in cells with reduced levels of HDAC1 (Figure 8F). Taken together, these studies showed that removal of the Sp5–HDAC1 complexes from the p21 promoter activates the promoter of p21 and increases protein levels of p21.

Discussion

The loss of hepatocyte markers is a critical step in the development of liver cancer. In this article, we identified mechanisms that are involved in the repression of hepatocyte markers and the p21 protein in hepatoblastoma. These occur on the levels of epigenetic repression of the promoters and are mediated by HDAC1 in complexes with the oncogenic form of C/EBPα and Sp5. The oncogenic form of

Figure 8. Reduction of Sp5–HDAC1 complexes by SAHA and inhibition of HDAC1 by small interfering RNA increases the expression of p21 protein. (A) Levels of Sp5 mRNA were determined by qRT-PCR in HeLa, HepG2, and Huh6 cells. (B) Co-IP studies show Sp5–HDAC1 complexes in HepG2 and Huh6 cells. Two repeats were performed. (C) Western blot shows levels of p21 as ratios to β-actin. *P < .05. (D) Treatment of HepG2 cells with a combination of LB100 + SAHA destroys Sp5–HDAC1 complexes. The SEC approach was used as described in the Figure 5D legend. (E) ChIP assay with p21 promoter. (F) Western blot shows that the inhibition of HDAC1 by small interfering RNA increases the expression of p21 in HepG2 cells. Box plots show ratios of p21 to β-actin. The experiment was performed 3 times with 3 biological replicates. LB, LB100; Un, untreated cells.
C/EBPα is created by dephosphorylation of Ser190 by PP2A. Similar to C/EBPα, PP2A initially was identified as a tumor-suppressor protein. Our data in this article strongly suggest that PP2A displays oncogetic activities in pediatric liver cancer. In support of our findings, several reports found that PP2A possesses oncogetic activities in other cancers. For example, HCC associated with hepatitis C infection expresses high levels of PP2A, and treatment of this HCC with inhibitors of PP2A reduce proliferation within these cells. Several studies have shown that an inhibitor of PP2A, cantharidin, has antitumor activity in many types of tumor cells. Our studies of HBL patients showed that HDAC1 and the oncogenic forms of C/EBPα and Sp5 are increased in tumor sections of HBL patients. Immunostaining of tumor sections of HBL patients showed that HDAC1 and C/EBPα are accumulated in dedifferentiating hepatocytes with intranuclear inclusions, which have been shown previously to contain the stem cell marker Delta Like Non-Canonical Notch Ligand 1 (DLK1). Further studies of patients with HBL showed that HDAC1 forms complexes with oncogenic C/EBPα and Sp5 and that these complexes occupy and repress promoters of hepatocyte markers CYP family proteins and the p21 promoter.

To test this hypothesis, we examined PP2A–C/EBPα–HDAC1 and Sp5–HDAC1 pathways in cancer cells derived from HBL patients: HepG2 and Huh6. The studies with inhibition of PP2A showed that PP2A is responsible for creating an oncogenic form of C/EBPα for formation of C/EBPα–HDAC1 complexes and subsequent repression of markers of hepatocytes. We also found that the olaparib–mediated reduction of HDAC1 and C/EBPα–HDAC1 complexes in PDX models leads to de-repression and increased expression of markers of hepatocytes. In tissue culture models, the inhibition of HDAC1 by SAHA leads to de-repression of the p21 promoter by removal of Sp5–HDAC1 complexes. Although SAHA inhibits activity of HDAC proteins, we surprisingly observed that SAHA also destroys Sp5–HDAC1 complexes and that Sp5 is not bound to p21 promoters after treatment. Thus, our studies of HBL patients, cells, and PDX models showed the critical role of HDAC1/C/EBPα/Sp5 in the development of pediatric liver cancer. In this regard, previous studies congruently have shown that the inhibition of PP2A additionally significantly inhibits the activity of HCC. The PP2A–C/EBPα pathway seems to be a critical part of PP2A oncogetic activities in liver cancer because restoration of C/EBPα activities by ectopic expression of constitutively active C/EBPα-S193D inhibits proliferation of cancer cells. In summary, given that LB100 is entering into clinical trials, as well as the inhibitor of HDAC1 SAHA, have significant potential for translational and clinical applications in treating HCC and HBL.

Methods

Animal

Animal experiments were approved by the Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital (protocol IACUC2014-0042).

Pediatric HBL Patients

This study underwent review and was approved before undertaking by the Institutional Review Board at Cincinnati Children’s Hospital Medical Center and University of Cincinnati Medical Center. Informed consent was obtained by each study patient before obtaining specimens. A significant portion of HBL samples were from patients who were faced with aggressive tumors that were characterized by multiple nodules at diagnosis, vascular invasion, chemoresistance, and relapse. These cases are referred to as aggressive hepatoblastoma.

Immunohistochemistry

Liver sections were fixed in 4% Paraphormaldehyde (PFA), embedded in paraffin, and sectioned (6-μm sections). Immunohistochemistry for HDAC1, C/EBPα, and HNF4α were performed with fresh HBL samples. Antibodies used were as follows: HDAC1 (05-100-I; EMD Millipore, Santa Cruz, CA), C/EBPα (sc-61; Santa Cruz, CA), and HNF4α (PP-K9218-00; Perseus Proteomics, Tokyo, Japan).

Real-Time qRT-PCR

Total RNA was isolated from human livers and from HepG2 and Huh6 cells as described. Complementary DNA was synthesized with 2 μg total RNA using a High-Capacity Complementary DNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA). Complementary DNA was diluted 5 times and subsequently used for RT-qPCR assays with the TaqMan Gene Expression system (Applied Biosystems). Gene expression analysis was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. Levels of all mRNAs were normalized to 18S ribosomal RNA. Data in this article present a summary of 3 technical replicates with 3–8 biological replicates. For qRT-PCR analyses of each gene, we used 22 background and 22 tumors sections of HBL patients.

Antibodies, Protein Isolation, Western Blot, Co-IP

Protein extracts were prepared as previously described. Nuclear extracts (50 μg) were loaded on 4–20% gradient gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes (Bio-Rad). For the investigations of PP2Ac, cytoplasmic nuclear and whole-cell extracts were used. Because C/EBPα is detected mainly in nuclei, Co-IP was performed with nuclear extracts using an improved Trueblot (Rockland Immunochemical, Limerick, PA) protocol as described. Antibodies used for Western blot and Co-IP were as follows: PP2Ac (clone7A6; Millipore Sigma, Burlington, MA), PPP2R2A (ab89621; Abcam, Cambridge, MA), PARP1 (Santa Cruz), cyclin D1 (sc-753; Santa Cruz), Gank (12985S; Cell Signaling Technologies, Danvers, MA), cdc2 (sc-954; Santa Cruz), CY3PA4 (sc-30621; Santa Cruz), CYP2B6 (sc-55924; Santa Cruz), CYP7A1 (E-10; Santa Cruz), PEPCK (F-3; Santa Cruz), HDAC1 (05-100-I; EMD Millipore), C/EBPα (sc-61; Santa Cruz), p21 (sc-6246; Santa Cruz), HNF4α (PP-K9218-00; Perseus Proteomics), Sp5 (ab36593; Abcam), and β-actin (A5316; Sigma). Antibodies
to ph-S190-C/EBPα have been described in our previous article. Western blot and Co-IP experiments were performed 3 times which each biological replicate.

**High-Pressure Liquid Chromatography–Based SEC**

To determine the size of C/EBPα–HDAC1 and Sp5–HDAC1 complexes, protein extracts were separated on high-pressure liquid chromatography SEC column SEC600 (Bio-Rad). A total of 30 μL from SEC fractions were loaded on denaturing 4%–20% acrylamide gel and examined by Western blot with antibodies to HDAC1, C/EBPα, and Sp5. For detection of protein–protein complexes in SEC fractions, C/EBPα or Sp5 were immunoprecipitated and HDAC1 was examined by Western blot using the improved Trueblot protocol. A detailed procedure of SEC has been described in our previous articles.

**ChIP Assay**

ChIP assay was performed as described in our previous articles. Briefly, chromatin solutions were isolated from background and tumor sections of livers of HBL patients. For detection of C/EBPα complexes, C/EBPα, HDAC1, p300, histone H3K9–Ac, and histone H3K9-trimethylated were immunoprecipitated and the IPs were examined by PCR with primers to CYP3A4 and PEPCK promoters. For Sp5–HDAC1 complexes, Sp5, HDAC1, H3K9Ac, and H3K9-trimethylated were immunoprecipitated from chromatin solutions. The IPs were examined by PCR with primers to p21 promoter, which cover 6 Sp binding sites. The sequences of the primers have been described in previous articles. This article shows representative results of 2–3 repeats of ChIP assays.

**Proliferation Assay**

Huh6 cells were seeded in 96-well plates at 5 × 10³. Cells were treated with LB100 (5 μmol/L), SAHA (1 μmol/L), and by a combination of these drugs. The Chokcystokin, CCK-8 reagent (Sigma) was added into each well and incubated for 4 hours, followed by reading at 450 nm on a microplate reader. Each experiment had 3 repeats per treatment and were repeated 3 times. The proliferation rate was calculated by comparing the absorbance of the treated and nontreated wells after 48 hours with the 0-hour nontreated well.

**Terminal Deoxynucleotidyl Transferase–Mediated Deoxyuridine Triphosphate Nick-End Labeling Assay**

A tunnel assay (ab66108; Abcam) was conducted with Huh6 cells treated with LB100, SAHA, and with a combination of LB100 + SAHA. Untreated and treated Huh6 cells were collected after 48 hours of treatment. After treatment, cells were harvested and fixed in 1% paraformaldehyde. Cells were resuspended in the DNA labeling solution consisting of reaction buffer, Terminal deoxynucleotidyl Transferase (TdT) enzyme, fluorescein isothiocyanate–deoxyuridine triphosphate, and ddH₂O and incubated in the DNA labeling solution for 60 minutes at 37°C. Cells were counterstained with propidium iodide/RNase A. The number of apoptotic cells was counted per each plate and presented as a box plot (Figure 6).

**Statistical Analysis**

All values are presented as means ± SEM. An unpaired Student t test was applied for comparison of normally distributed data. Two-way analysis of variance was used with a Bonferroni test for multiple comparisons between different time points if the P value was less than 0.05. Statistical significance was defined as follows: P < 0.05, P < .01, P < .001, and P < .0001. All statistical analyses were performed using GraphPad Prism 6.0 (San Diego, CA).

All authors had access to the study data and approved the final manuscript.

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The authors disclose no conflicts.

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