Aryl hydrocarbon receptor regulates histone deacetylase 8 expression to repress tumor suppressive activity in hepatocellular carcinoma

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Keywords: AHR, HDAC8, hepatocellular carcinoma (HCC)

Received: February 05, 2016 Accepted: May 20, 2016 Published: June 06, 2016

ABSTRACT

Histone deacetylase 8 (HDAC8), a unique member of class I histone deacetylases, shows remarkable correlation with advanced disease stage and multiple malignant tumors. However, little is known about the contribution of HDAC8 to the tumorigenesis of hepatocellular carcinoma (HCC). The present study investigated the expression of HDAC8 regulated by the aryl hydrocarbon receptor (AHR) in HCC cell lines and tissues, and the roles of HDAC8 overexpression in cell proliferation, including potentially underlying mechanisms. We assessed the correlation between the clinic-pathological parameters and the expression of AHR and HDAC8. Further, we analyzed the AHR siRNA transfection and HDAC8 inhibitors to explore the functions of HDAC8 in HCC progression in vitro and in vivo. In a panel of 289 HCC patients, HDAC8 was shown to be highly correlated with AHR expression at both mRNA and protein levels. HCC patients with high AHR expression showed a shorter survival time than that with low AHR expression. We then found that the expression of both AHR and HDAC8 was significantly upregulated in both HCC cell lines and tumor tissues compared to human normal hepatocytes and matched non-tumor tissues. Furthermore, HDAC8 inhibition remarkably inhibited hepatoma cell proliferation and transformation activity via upregulation of RB1 in vitro and in vivo. Our data revealed an important role of the AHR-HDAC8 axis in promoting HCC tumorigenesis, thus identifying HDAC8 as a potential therapeutic target for HCC treatment.

INTRODUCTION

The liver is the major organ involved in the metabolism of xenobiotics resulting from environmental pollution or dietary intake. A multitude of xenobiotics and their metabolites have detrimental and tumorigenic effects on hepatocytes and may cause liver tumor formation [1]. The aryl hydrocarbon receptor (AHR),
a unique cellular chemical sensor in most organs, is a cytosolic basic helix–loop–helix/Per–Arnt–Sim transcriptional factor [2]. It can be activated by numerous environmental pollutants, including polycyclic aromatic hydrocarbons and a diverse set of endogenous metabolites [3, 4]. AHR resides in the cytoplasm in its resting state and upon encountering ligands, it is translocated to the nucleus where it heterodimerizes with the AHR nuclear translocator (ARNT) [5] and activates the expression of a battery of genes containing specific DNA-enhancer sequences, which are known as xenobiotic-responsive elements (XREs) [6]. These downstream genes include those encoding phase I and phase II enzymes [7] that are involved in the cellular detoxification process, where toxic and carcinogenic metabolites can be generated, which may play an important role in multiple stages of tumor progression in humans and experimental models [2, 7]. In addition to cellular detoxification, AHR is known to regulate signal transduction pathways involved in cellular metabolism, proliferation, differentiation, and apoptosis. Disruption of the AHR cellular functions is also associated with the expression of various diseases [7, 8]. Several studies have shown that dysregulation of AHR in hepatocytes leads to steatosis and aberrant cholesterol metabolism [9–11], and increased expression of AHR and its binding partner, ARNT, has been noted in hepatocellular carcinoma (HCC) [12]. However, the pathogenic mechanisms and the possible prognostic value of AHR expression in HCC are yet to be elucidated.

Histone deacetylases (HDACs), a family of enzymes with the ability to remove acetyl groups from lysine on histones and other proteins to repress downstream gene expression by wrapping the DNA more tightly, substantially contribute to the onset and progression of human diseases [13, 14]. In humans, HDACs are grouped in four classes of proteins: class I, IIa, Ib, III, and IV [15–17]. HDAC8, a class I zinc-dependent HDAC, typically induces histone deacetylation and represses gene transcription [18, 19]. HDAC8 is restricted to specific cell types exhibiting smooth muscle differentiation in normal human tissues. Loss of HDAC8 activity has been shown to result in increased SMC3 acetylation and inefficient dissolution of cohesin complexes [20]. Aberrant upregulation of HDAC8 was suggested to be correlated with NAFLD-associated HCC development [21]. Although HDAC8 has been shown to promote growth of numerous cancer types and contribute to poor prognosis in childhood neuroblastoma [22–24], the molecular actions of HDAC8 in cancer remained poorly defined.

In this study, we provide evidences that suggest a plausible mechanism linking AHR and HCC via targeting of HDAC8, which promotes tumor cell growth and may restrain the expression of RB1 tumor suppressors in HCCs.

RESULTS

HDAC8 showed tumor-specific expression pattern correlated with HCC clinical outcome

AHR, a major chemical sensor receptor, is known to regulate signal transduction pathways involved in cellular metabolism, proliferation, differentiation, and apoptosis [3, 4]. Disruption of the cellular functions of AHR is also associated with the expression of various diseases, including HCC [25]. The underlying regulatory mechanisms of tumorigenesis remain unclear. To investigate the mechanism of the oncogenic action of AHR, we analyzed the gene expression patterns in paired samples of tumors from the high and low AHR group by microarray analysis. The expression of epigenetic genes, HDAC8, was elevated in high-AHR HCC tumors but decreased in the tumor suppressor genes RB1 and p53 (Figure 1A). To investigate the correlation and clinical outcome between the expression of AHR and HDAC8, the mRNAs for these genes were examined in a panel of 289 paired HCC tumor samples (tumor paired with neighbor “healthy” liver tissues). Analysis of the expression patterns of HDAC8 in various tissue samples first showed that HDAC8 mRNA expression in HCC liver samples was significantly upregulated as compared with that in non-HCC patients (Figure 1B; p = 0.0008, one-way ANOVA), while the expression pattern of HDAC8 in HCC samples varied greatly. In fact, analysis of the dichotomized group with the HDAC8 expression at the top 20 percentile (n = 56; “high HDAC8”) showed distinct clinical features compared with those of the remaining HCC patients (n = 230; “low HDAC8”). As shown in Table 1, when the two groups and the non-HCC subjects were compared, significant differences were observed in the levels of albumin (p = 0.0062) and lymph vascular invasion (p = 0.0163) as well as in tumor size (p = 0.002), tumor number (p = 0.0429), and tumor grade (p = 0.001), but not in age, sex, or the levels of GOT, alkaline phosphatase, trypgliceride, g-GT, AC sugar, bilirubin, and cholesterol (Table 1). Further, HDAC8 expression in tumor samples showed a tumor-specific expression pattern in HCC tumor masses (indicated in brown; T) compared with the adjacent healthy liver tissues (N) and negative mouse IgG (Supplementary Figure S1), and HDAC8 expression was detected in both the cytoplasm and nucleus (yellow arrow) of tumor cells (Figure 1C). The analysis of the survival curves of HCC patients showed a significantly shorter survival time after surgical resection for patients in high HDAC8 groups than in the low HDAC8 expression groups (Figure 1D, p = 0.0004).
Figure 1: HDAC8 showed a tumor-specific expression pattern and strong correlation with the clinical outcome of HCC patients. A. Heat map analysis of cDNA microarray data of “high” AHR HCC tumors compared with data from adjacent samples of healthy liver tissue. High HDAC8 mRNA expression was detected in HCC tumors with high AHR expression. B. HDAC8 mRNA was overexpressed in HCC tumor samples compared with non-HCC liver samples. C. HDAC8 expression showed a tumor-specific pattern in HCC tumor samples. HDAC8, brown; HDAC8 (nuclei), yellow arrow; N, adjacent healthy liver tissue. D. High HDAC8 mRNA expression in HCC is associated with a shorter survival time in HCC patients than that associated with low HDAC8 mRNA expression (Kaplan–Meier survival analysis). E. HDAC8 expression showed a high correlation with AHR mRNA expression. The HDAC8 mRNA expression in HCC tumors with high AHR mRNA expression was significantly higher than that in tumors with low AHR mRNA expression.
Table 1: Baseline characteristics of 289 hepatocellular carcinoma (HCC) patients and 243 non-hepatocellular carcinoma patients

<table>
<thead>
<tr>
<th>Group</th>
<th>Non-HCC (n=243) (%)</th>
<th>HDAC8 mRNA (Low, n=230)(%)</th>
<th>HDAC8 mRNA (High; n=59)(%)</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>Age (mean(SD))</td>
<td>61.6±5.04</td>
<td>57.9±2.3</td>
<td>59.9±1.4</td>
<td>0.6851</td>
</tr>
<tr>
<td>Sex</td>
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<tr>
<td>Male</td>
<td>154(63.37)</td>
<td>164(71.30)</td>
<td>48(81.36)</td>
<td>0.6017</td>
</tr>
<tr>
<td>Female</td>
<td>89(36.63)</td>
<td>66(28.70)</td>
<td>11(18.64)</td>
<td></td>
</tr>
<tr>
<td>GOT (U/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40</td>
<td>214(88.06)</td>
<td>94(40.87)</td>
<td>19(32.20)</td>
<td>0.2670</td>
</tr>
<tr>
<td>40≤&lt;100</td>
<td>16(6.58)</td>
<td>102(44.35)</td>
<td>26(44.07)</td>
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</tr>
<tr>
<td>100≤</td>
<td>13(5.36)</td>
<td>34(14.78)</td>
<td>14(23.73)</td>
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<tr>
<td>GPT (U/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40</td>
<td>216(88.89)</td>
<td>85(36.96)</td>
<td>21(35.59)</td>
<td>0.8844</td>
</tr>
<tr>
<td>40≤&lt;100</td>
<td>14(5.76)</td>
<td>106(46.09)</td>
<td>26(44.07)</td>
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<tr>
<td>100≤</td>
<td>13(5.35)</td>
<td>39(16.95)</td>
<td>12(20.34)</td>
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<td>&lt;4.5</td>
<td>219(90.12)</td>
<td>195(84.78)</td>
<td>57(96.61)</td>
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<td>≥4.5</td>
<td>24(9.88)</td>
<td>35(15.22)</td>
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<td>&lt;20</td>
<td>218(89.72)</td>
<td>130(56.52)</td>
<td>34(57.63)</td>
<td>0.8909*</td>
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<td>≥20</td>
<td>25(10.28)</td>
<td>100(43.48)</td>
<td>25(42.37)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1.5&lt;</td>
<td>132(54.32)</td>
<td>189(82.17)</td>
<td>50(84.75)</td>
<td>0.8223*</td>
</tr>
<tr>
<td>≥1.5</td>
<td>111(45.68)</td>
<td>41(17.83)</td>
<td>9(15.25)</td>
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<td>Lymphovascular invasion</td>
<td>243(100)</td>
<td>165(71.74)</td>
<td>32(57.14)</td>
<td>0.0163*</td>
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<tr>
<td>No</td>
<td>0</td>
<td>65(28.26)</td>
<td>27(45.76)</td>
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</tr>
<tr>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size(cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2.5</td>
<td>82(35.65)</td>
<td>8(13.56)</td>
<td>0.0020*</td>
<td></td>
</tr>
<tr>
<td>2.5≤</td>
<td>148(64.35)</td>
<td>51(86.44)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of tumors</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>181 (78.70)</td>
<td>39(66.10)</td>
<td>0.0429*</td>
<td></td>
</tr>
<tr>
<td>1&lt;</td>
<td>49(21.30)</td>
<td>20(33.90)</td>
<td></td>
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</tr>
<tr>
<td>Modified TNM</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>153(66.52)</td>
<td>23(38.98)</td>
<td>&lt;0.001*</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>61(26.52)</td>
<td>20(33.90)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III(IIIA and IIIB)</td>
<td>16(6.96)</td>
<td>16(27.12)</td>
<td></td>
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</table>

*p values were calculated by Fisher exact test

High HDAC8 mRNA expression patients: HDAC8 mRNA expression in tumor part were higher three point six folds (>3.6 folds) than adjacent normal part liver tissue.

Low HDAC8 mRNA expression patients: HDAC8 mRNA expression in tumor part were lesser three point six folds (<3.6 folds) than adjacent normal part liver tissue. (cut point by survival ROC curve)

*p<0.05.

Patients: 289HCC patients from two medical centers [Chung Ho Memorial Hospital (255HCC) and Changhua Christian Hospital (34 HCC)] were enrolled into the HDAC8 cohort study from July 2007 to July 2015.
**HDAC8 mRNA expression correlated with AHR expression**

The expression of *HDAC8* mRNA strongly correlated with the expression of *AHR* in HCC patients (Person’s correlation coefficient, $r = 0.7504$, $p < 0.001$; Figure 1E). In 8 randomly selected HCC samples obtained from patients in the high *AHR* group, the increased *HDAC8* expression correlated with the expression pattern of *AHR* when compared with those of paired tumor-adjacent, normal tissues (Figure 2A). The expression correlation between AHR and HDAC8 was further verified by immunofluorescence staining of HCC patient tumor tissue. High level of AHR expression was noted in tumor cells co-expressing high level of HDAC8 (Figure 2B). *HDAC8* mRNA expression in tumors of the “high AHR” group was significantly higher than that in the “low AHR” group (Figure 2C). Analysis of the dichotomized group (“high AHR” vs. “low AHR” expression) placed the expression of *HDAC8*, *p53*, *RB1*, *E2F1*, and *CCND1* in the top 25 percentile ($n = 57$; “high AHR”). *HDAC8* expression and expression of *CCND1* and *E2F1* in tumors of the “high AHR” group were significantly higher than in the “low AHR” group ($n = 232$; “low AHR”; Figure 2D).

![Figure 2: Ectopic HDAC8 expression showed a high correlation with AHR in hepatocellular carcinoma (HCC).](image)

- **A.** Overexpression of HDAC8 protein was detected in HCC patients with high AHR mRNA expression.
- **B.** The expression pattern of HDAC8 showed significant co-localization with AHR in tumor cells of HCC patients. HDAC8, red; AHR, green.
- **C.** High levels of HDAC8 mRNA were detected in HCC patients exhibiting high AHR mRNA expression.
- **D.** High AHR expression coupled with high proto-oncogenes (*CCND1* and *E2F1*) expression but with lower tumor suppressor gene (*p53* and *RB1*) expression in HCC patients. a, six samples from the high AHR group could not be detected.
AhR expression construct to identify the regulatory promoter region that is controlled by AHR (Figure 3F). AHR binds in the potential transcriptional regulatory elements to which HDAC8 are highly expressed in all of the hepatoma cell lines as compared with expression levels in normal hepatocytes (Chang normal liver cells, CNL; Figure 3A). Moreover, when hepatoma cells (SK-Hep1) were treated with TCDD, a prototypical AhR ligand, expressions of HDAC8 and the classical AhR target gene CYP1B1 were increased in hepatoma cells in a time-dependent manner, both at protein level (Figure 3B). To determine whether AHR activated HDAC8 via a genetic mechanism, we transfected an AHR expression construct into hepatoma cells, and the mRNA and protein levels of HDAC8 were measured by real-time PCR and western blotting, respectively, in AHR-overexpressing cells. As shown in Figure 3C and 3D, forced AHR overexpression enhanced mRNA and protein expression of CYP1B1 and HDAC8 in both hepatoma cell lines. The protein level of HDAC8 was also increased when AHR-GFP overexpression (induced by a Tet-on system) was induced by different concentrations of doxycycline (0.3 and 1 μg/mL) for 8 h (Figure 3E). Interestingly, reduction in the expression levels of two tumor suppressors, RB1 and p53, was also noted in AHR-overexpressing hepatoma cells. This was consistent with the finding that in HCC clinical samples, the expression levels of RB1 and p53 were significantly lower in the “high AhR group” in comparison to those in the “low AhR group,” suggesting the existence of an inverse relationship between high and low AhR expression of these two tumor suppressors.

To further explore and verify the regulatory effects of AHR on HDAC8 expression, we used a promoter assay and chromatin immunoprecipitation (ChIP) to analyze the potential transcriptional regulatory elements to which AHR binds in the HDAC8 promoter. A HDAC8 promoter with serial deletions was inserted into a luciferase expression construct to identify the regulatory promoter region that is controlled by AHR (Figure 3F). AHR significantly increased HDAC8 promoter–driven luciferase activity (11.89 ± 1.74) of the expression constructs with a promoter sequence longer than 168 bp (Figure 3F). Analysis of the HDAC8 promoter region between positions −168 and +30 identified one classical XRE (T/GNGCGTGA/CG/CA) in the locus comprising −108 to −125. The transactivation effect of AHR on the XRE element of the HDAC8 promoter was further confirmed by a promoter binding assay. As shown in Figure 4A, the HDAC8 promoter regions (positions −168 to +30) were pulled down by an anti-AHR polyclonal antibody in SK-Hep1 hepatoma cell lines transfected with AHR. In contrast, the HDAC8 promoter region was not pulled down if XRE was deleted (Figure 4A). The AHR binding to HDAC8 promoter regions in vivo were analyzed using ChIP analysis. The HDAC8 promoter region between −168 and +30 bp was pulled down by the anti-AHR antibody in hepatoma cells with high HDAC8 expression (Figure 4B).}

**HDAC8 downregulated RB1 expression via direct promoter binding**

To explore the potential functional relationship between HDAC8 and RB1, analyses of the expression patterns of HDAC8 and RB1 were first examined in hepatoma cell lines. An HDAC8 expression construct was transfected into hepatoma cells, and the levels of RB1 were measured by Western blotting in HDAC8-overexpressing cells. As shown in Figure 4C, forced HDAC8 overexpression suppressed protein expression of RB1 in hepatoma cell lines. Also, the RB1 promoter regions (positions −250 to +30) were pulled down by the anti-HDAC8 polyclonal antibody in SK-Hep1 hepatoma cell lines transfected with GFP-tagged HDAC8 (Figure 4D). Our data showed that the induction of AHR activated cellular expression of HDAC8 and consequently suppressed RB1 expression in hepatoma cells. However, it remained unclear whether the expression of HDAC8 occurs in an AHR-dependent manner under physiological conditions. To address this question, the expression of Amt1, Cyp1b1, and Hdac8 was analyzed in hepatocytes from AhR-wild type (AhR+/+) or–null (AhR−−) mice. Results revealed dramatically reduced protein and mRNA levels for all parameters in AhR-null hepatocytes compared with those of AhR+/− or AhR−− cells (Figure 4E). In fact, hepatocytes from AhR−− heterozygotes also showed lower levels of gene expression when compared with those of AhR-wild type cells (Figure 4E). To test if the inhibition of RB1 and HDAC8 expression was directly affected by AhR, hepatocytes isolated from AhR-wild type and AhR-null mice were treated with dioxin. As shown in Figure 4F, increased HDAC8, but decreased Rb1, expression was found in dioxin-treated AhR-wild type primary hepatocytes but not in those from AhR-null mice. The modulation regulatory effect of RB1 by HDAC8 was finally determined in hepatoma cells with ectopic HDAC8 expression but transfected with AHR shRNAi. The hepatoma cells (SK-Hep1) with ectopic HDAC8 expression reversed the enhancing effects of AHR knockdown on the expression of RB1 and p53 (Figure 4G). These results indicated that HDAC8 are direct physiological targets of AhR and that Rb1 in turn regulates cellular proliferation as a consequence of AhR activation.
The AHR-HDAC8 axis regulated tumor proliferation and transformation in vitro and in vivo

To explore the potential tumorigenic activity of the AHR-HDAC8 axis, an inducible expression system overexpressing AHR, a constitutively activated AHR mutant (CA1) [10], and HDAC8 was tested in two HCC cell lines (Hep 3B and SK Hep1). After treatment of the cells with doxycycline (1 µg/mL) for 8 h, overexpression of AHR or the CA1 mutant significantly enhanced BrdU incorporation by 47.9% and 97.8%, respectively, as

Figure 3: AHR directly activated cellular HDAC8 expression via the AHR–ARNT complex in hepatoma cells. a, p < 0.001. 
A. AHR expression in hepatoma cell lines showed strong correlation with the expression of HDAC8 in Hep G2, Hep 3B, SK-Hep1, Huh 7, PLC/PRF/5, and HA22T are HCC cell lines. B. AHR activation induced by TCDD increased cellular CYP1B1 and HDAC8 protein levels in hepatoma cells. C. Forced AHR expression increased the mRNA expression of the downstream genes CYP1B1 and HDAC8. D. Forced AHR expression increased the protein expression of the downstream genes CYP1B1 and HDAC8. Forced AHR and ARNT1 showed an additive effect on HDAC8 expression. E. AHR expression induced by doxycycline increased HDAC8 expression but decreased p53 and RB1 expression in hepatoma cells. F. AHR activated luciferase activities driven by the HDAC8 promoter until promoter regions less than −168 bp.
compared with those transfected with the empty-vector control (Figure 5A). The hepatoma cells with HDAC8 expression showed a lesser degree of enhancement (30%) of BrdU incorporation than those overexpressing AHR. Hepatoma cells with both AHR and HDAC8 expression showed an additive effect on cell proliferation activity. Enhancement of cellular transformation activity by AHR, CA1, and HDAC8 in HCC cells was observed in an independent soft agar assay (AIG), wherein overexpressed AHR, CA1 mutant, and HDAC8 significantly increased colony formation by 54%, 78%, and 42%, respectively, after induction by doxycycline (Figure 5B). The hepatoma cells exhibiting both AHR and HDAC8 expression showed a more pronounced additive effect on transformation activity than that of AHR or HDAC8 alone.

To further evaluate the role of AHR-HDAC8 signaling in the proliferation and tumorigenesis of HCC, hepatoma cells treated with HDAC8-specific inhibitors, PCI-34051, and 1-Naphthohydroxamic acid (1-NTHX) [26, 27], showed enhanced expression of RB1 and p53, as well as a lower level of proliferative activity (Figure 5C and 5D). The tumor growth in vivo of hepatoma cells was also reduced in nude mice treated with the HDAC8-specific inhibitor PCI-34051 (5 μM) for four weeks (Figure 5E), and the tumor mass in mice treated with PCI-34051 showed lower levels of

Figure 4: AHR physiologically regulates HDAC8 expression in normal mouse hepatocytes. a, p < 0.001. A. The HDAC8 promoter with the −108 to −125 bp deletion abolished the promoter binding activity of AHR. B. Chromatin HDAC8 promoter binding activity showed an AHR-dependent increase in hepatoma cells. The HDAC8 promoter binding activity of the AHR complex in hepatoma cells determined with ChIP in vivo. C. Forced HDAC8 downregulated RB1 expression. D. Forced HDAC8 expression in hepatoma cells enhanced the chromatin binding activity of HDAC8 on the RB1 promoter. E. HDAC8 expression in hepatocytes was abrogated in AhR-null mice. F. The ablation of HDAC8 expression in hepatocytes showed involvement of AHR-dependent regulation. G. The hepatoma cells (SK-Hep1) transfected with HDAC8 showed to downregulate the enhancement on tumor suppressors, p53 and RB1, expression induced by cells transfected with AHR shRNAi.
proliferation marker (Cyclin D1, E2F1, PCNA and Ki 67) expression than those treated with DMSO (0.1%) (Figure 5F) [28]. These results indicated that AHR and its target, HDAC8, play an important role in terms of promoting tumorigenesis of HCC.

**DISCUSSION**

AHR, a unique chemical sensor activated by a large number of xenobiotics, has been considered a major regulator of xenobiotic-induced carcinogenesis,
but its role in the processes of tumor initiation and development remains to be elucidated. In this study, we showed that the inactivation of tumor suppressor RB1 occurred by activation of AHR-HDAC8-axis signaling. In a panel of 289 HCC patients, HDAC8 was shown to be highly correlated with AHR expression at both mRNA and protein levels. Genetically, AHR activated HDAC8 expression via direct binding to the HDAC8 promoter region and consequently downregulated tumor suppressor RB1 expression. These results clearly show why chronic exposure of toxic environmental molecules may result in the development of hepatic neoplasia or malignant tumors.

Cancer is primarily an environmental disease with 90%–95% and 5%–10% of the cases being attributed to environmental and genetic factors, respectively [29]. Despite extensive research, environmental risk factors of cancer remain largely unknown. Many studies have shown that dysregulation of AHR in hepatocytes leads to steatosis and aberrant cholesterol metabolism [10], and increased expression of AHR has been noted in HCC [12], suggesting its potential pro-tumorigenic activity; however, the tumorigenic activity of AHR is still unclear. In this study, we provided further evidence that AHR expression influences HCC tumorigenicity by suppressing the expression of tumor suppressor RB1. This difference in genetic outcomes between the “low” and “high” AHR groups appeared to be determined by the change in expression levels of tumor the suppressor genes p53 and RB1 and of those of the proto-oncogenes HDAC8, CCND1, and E2F1. Evidence from our clinical data and analysis of tumor cells showed that high AHR expression directly enhanced its downstream gene-HDAC8 expression, which led to low expression of tumor suppressors (p53 and RB1); however, it was associated with higher levels of expression for proto-oncogenes (CCND1 and E2F1) in vitro and in vivo. The results provided mechanistic evidence that the tumorigenic activation induced by high AHR expression is involved in HCC formation, suggesting a potential target for HCC therapy.

Inactivation of tumor suppressor activity of e.g., RB1 and p53 is the most frequent hallmark of cancer, including HCC [28, 30, 31]; however, the detailed regulatory mechanisms are still largely unknown. RB1, a classic tumor suppressor, is frequently inactivated in many malignancies. In general, according to cancer genome studies, RB1 was genetically inactivated in about 22% of patients with prostate cancer [32]. Evidence also indicates that the RB pathway is functionally inactivated in most cases of HCC by genetic, epigenetic, and/or viral mechanisms [33]; the activation of the RB pathway in a mouse model may prevent HCC development by maintaining the quiescence of adult liver progenitor cells [28, 34]. However, as evidenced by SNP analysis, genetic mutations of the RB1 gene are relatively rare and few studies were focused on the inactivation of RB signaling in HCC. Recently, a systematic screen for the identification of imprinted genes deregulated in human HCC revealed that RB1 shows imprint abnormalities in a high proportion of primary patient samples [35, 36]. Forty percent of the HCC specimens (16/40) showed hyper- or hypo-methylation at the CpG island in intron 2 of the RB1 gene [35]. In this study, we first showed that, beside methylation, promoter deacetylation of the RB1 gene induced by HDAC8 represents another potential important mechanism for RB1 inactivation in human HCC, complementing already well-described molecular defects. These results provide advanced epigenetic evidence of acetylation in RB1 expression, a potential therapeutic target in HCC.

In summary, we demonstrated that AHR regulated cell proliferation and tumorigenesis by directly targeting and activating HDAC8 expression in hepatoma cells. The strong correlation of mRNA and protein levels between AHR and HDAC8 in patients with HCC underscores the important regulatory role of the AHR signaling axis in HCC progression, suggesting its potential utility as a new therapeutic target and for developing preventive measures in HCC.

MATERIALS AND METHODS

Patients

This study enrolled 289 patients with hepatocellular carcinoma (HCC) from July 2007 to July 2015 from two medical centers (Chung Ho Memorial Hospital [255 patients] and Changhua Christian Hospital [34 patients]) with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) infection and 243 non-HCC patients, of whom 85 were infected with HBV or HCV and 68 were not infected with either virus. Of these patients, 289 had adequate follow-up data for detailed analysis. The study of human subjects was approved by the Institutional Review Board of Kaohsiung Medical University (KMUHIRB-20130052; Kaohsiung, Taiwan).

Animals and cell culture

Male BALB/c nu/nu mice were obtained from the National Laboratory of Animal Breeding and Research Center (Taipei, Taiwan) and housed according to the protocols of the Animal Center, Kaohsiung Medical University, Kaohsiung, Taiwan [37]. Normal hepatocyte cells (Chang normal liver cells; CNL) and human hepatoma cell lines (HepG2, Hep3B, SK-Hep1, Huh7, PLC/PRF/5, HCC 36 and HA22T) were obtained from American Type Culture Collection (Manassas, VA, USA) and maintained according to the manufacturer’s protocol [38].

Plasmids, cell lines, and other materials

Full-length AHR cDNA was amplified using PCR from a human testis cDNA library (GIBCO/BRL). The
AHR cDNA and a mutant CAI were each subcloned into the pEGFP/C1 (pCDNA3/Ha) vector (Clontech) or a Tet-on system (pAW4.puro, NCFPB, Taiwan) to express a GFP (Ha)-tagged AHR. The PLKO.1.puro or neo vector was used as a backbone for shRNAi constructs targeting AHR and HDAC8 (NCFPB, Taiwan), SK-Hep1, Hep G2, Hep 3B, HA 22T, PLC cell lines were sub cultured and maintained according to American Type Culture Collection (ATCC) protocols. Transfection was performed using the Lipofectamine transfection kit (GIBCO/BRL).

Western blotting and immunohistochemical analysis

Western blotting and immunohistochemical (fluorescence) staining were performed as previously described [37, 38]. The primary antibodies used in this study were AHR (RPT9), HDAC8 (GeneTex), RB1, p53, Cyclin D1, PCNA, ARNT1, CYP1B1 and E2F1 (1:200), actin polyclonal antibodies (1:5000 dilution; Sigma–Aldrich), HA and GFP monoclonal antibodies (1:200 dilution; Upstate, NY, USA), FITC-conjugated anti-rabbit IgG, rhodamine-conjugated anti-mouse IgG, alkaline phosphatase-conjugated anti-rabbit IgG antibody (1:500 dilution; Vector Laboratories), and Ki67 goat polyclonal antibody (1:200 dilution; Santa Cruz Biotechnology) [28]. All of the experiments were repeated at least three times, and staining with relevant control antibodies was performed in parallel and in all cases no signals were seen (for example, Supplementary Figure S1).

Luciferase reporter assays

The HDAC8 promoter (between positions –1101 and +30 bp) was cloned from human placenta genomic DNA and was used to construct a pGL3 luciferase reporter plasmid [28, 38]. The expression constructs and two reporter constructs, pSV40-Rluc and pGL3-HDAC8/Fire luciferase (Promega), were co-transfected with pEGFP-AHR into 2 × 10⁴ Hep 3B cells. The cells were harvested 16 h after the transfection, and the relative luciferase activity was measured according to the manufacturer’s instructions. All data are expressed as the mean ± SD of at least three experiments.

Chromatin immunoprecipitation (ChIP) assays

Chromatin immunoprecipitation (ChIP) assays were performed as described previously [38]. All data are expressed as the mean ± SD of at least three experiments. An HDAC8 promoter fragment was amplified with the following primers: Primer 1, 5′-TTC ACT GGG GTT CCG AG-3′ and Primer 2, 5′-TGC CCC AAG CCT CCG GA-3′. An RB1 promoter fragment was amplified with the following primers: Primer 1, 5′-TTG CCA TTG CTG CTG C-3′ and Primer 2, 5′-CGT CCT CCC AAA CCA TTC A-3′.

Real-time PCR

The expression of AHR and HDAC8 mRNA in hepatoma cells and cells from cancer patients were quantified using the SYBR Green Quantitative RT-PCR kit (Invitrogen) as described previously. Total RNA was extracted from the tumor mass using TRIzol reagent (Invitrogen) and transcribed into cDNA (Invitrogen) for PCR amplification using a 7900HT Thermocycler (Applied Biosystems Inc.). All procedures and data analyses were performed according to the manufacturer’s instructions. Data on cells transfected with an empty pEGFP vector and samples from healthy subjects and drug-treated patients were analyzed and compared. All data are expressed as the mean ± SD of at least three experiments.

Anchorage-independent growth assays

Cells (10⁴ or 5 × 10⁴) in 1 mL of culture medium were mixed with an equal volume of 0.6% top agar and plated onto 60-mm culture dishes with 0.5% bottom agar [37, 38]. The plates were incubated at 37 °C for 2 weeks. Colonies were visualized by staining with 0.05% crystal violet acetate, and only those larger than 0.5 mm were counted. The culture medium was replaced every 3 days. All data are expressed as the mean ± SD of at least three experiments.

Assessment of the tumorigenic properties of HDAC8 in nude mice

Male BALB/c nu/nu mice were inoculated (s.c. injection) with 10⁴ of either scrambled vector or HDAC8-GFP-transfected overexpression cells on both sides of the back (10 mice per group) [37, 38]. Tumor volume was measured using a caliper once or twice a week. Tumor volume was estimated according to the formula: volume (cm³) = (L × W²)/2, where L and W represent the length and width of the tumor, respectively. Tumor sizes were presented as means ± SD.

Statistical analysis

The quantitative variables are presented as means ± SD. The statistical significance of differences was determined using a two-sample t-test. Pearson’s correlation analysis was used to examine the relationship between expression levels of AHR and HDAC8. Statistical analysis of categorical variables was performed using chi-squared analysis, one-way analysis of variance (ANOVA), and Fisher’s exact test. Differences with a p value < 0.05 were considered statistically significant.
ACKNOWLEDGMENTS

None.

FUNDING

This work was supported in part by research grants NSC-102-2320-B-037-032-MY3 from the National Science Council, Taiwan, EOPP10-014 from the Ministry of Science and Technology and National Science Council, Taiwan. This work was supported, in part, by grants from National Health Research Institutes, Taiwan (EOPP10-014 andEOSP07-014). This study is also supported partially by Top Universities Grant, grant No. KMU-TP-104E10 and KMU-TP-104A10 from Kaohsiung Medical University.

CONFLICTS OF INTEREST

The authors have no conflicts of interest.

REFERENCES


