Activation of IL6/IGFIR Confers Poor Prognosis of HBV-Related Hepatocellular Carcinoma through Induction of OCT4/NANOG Expression

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Abstract

Purpose: To unravel the role of interleukin (IL)-6 and insulin-like growth factor (IGF)-1 receptor (IGFIR) in expressing stemness-related properties and to evaluate the prognostic values of pluripotent transcription factor OCT4/NANOG, and IGFIR in hepatocellular carcinoma (HCC).

Experimental Design: Serum levels of IL6 were detected using ELISA assays (n = 120). The effects of IL6/IGF on stemness expression in HCC were examined using OCT4/NANOG promoter luciferase reporter, RNA interference, secondary sphere formation, side population, and xenograft animal models. The OCT4/NANOG protein and phospho-IGFIR receptor (p-IGFIR) in tissues were detected by Western blotting (n = 8) and immunohistochemical staining (n = 85). OCT4, NANOG, and IGFIR expression levels in tissues (n = 191) were analyzed by real-time qRT-PCR and was correlated with early tumor recurrence using the Kaplan–Meier survival analysis.

Results: A high positive correlation between the expression levels of OCT4/NANOG and IGFIR/p-IGFIR in human HCC tissues was observed. The concurrent expression of OCT4/NANOG/IGFIR was mostly confined to hepatitis B virus (HBV)–related HCC (HBV-HCC) and was significantly correlated with early tumor recurrence. High serum levels of IL6 were significantly correlated with high OCT4/NANOG expression. IL6 stimulated an autocrine IGF/IGFIR expression STAT3 dependent-ly, which stimulated stemness-related properties in both the cell lines and the xenografted mouse tumors. The inhibition of IGFIR activation by either RNA interference or by treatment with the inhibitor picropodophyllin (PPP) significantly suppressed the IL6-induced stemness-related properties both in vitro and in vivo.

Conclusions: The expression of pluripotency-related genes is associated with early tumor recurrence and is regulated by IL6-induced IGF/IGFIR activation, particularly in HBV-HCC. Clin Cancer Res; 21(1); 201–10. © 2014 AACR.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer, and the third leading cause of cancer-related mortality worldwide (1). Surgical resection can be curative in the early stages of HCC, but the risk of recurrence remains high. As tumor stemness properties are related to HCC recurrence (2), investigating the mechanisms responsible for the expression of tumor stemness could lead to strategies to prevent tumor recurrence and to improvements in the efficacy of HCC treatment.

Clinical and epidemiologic studies have identified a clear relationship between chronic hepatic inflammation and HCC pathogenesis (3). The inflammation associated with chronic hepatitis B virus (HBV) infection contributes to HCC (4). Following HBV infection, Kupffer cells activate nuclear factor-kB

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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and secrete inflammatory cytokines, such as tumor necrosis factor-α, interleukin (IL)-8, IL1β, and IL6 (5). Meanwhile, the HBV X protein (HBx) can stimulate IL6 expression in hepatocytes and HCCs (6). A clinical association between IL6 and HBV-related HCC (HBV-HCC) has been previously reported. It has been shown that IL6 controls early gene expression in HBV infection (5), and that a high serum level of IL6 can predict future HCC development in patients with chronic hepatitis B (7). It was recently suggested that aberrant IL6-mediated signaling can transform hepatic progenitor cells in HBx transgenic mice (8), and that aberrant IL6-mediated signaling is responsible for liver progenitor/stem cells giving rise to cancer (9). In addition, it has been demonstrated that CD24+ liver tumor-initiating cells can drive self-renewal and tumor initiation through STAT3-mediated NANOG regulation (10). These observations highlight the association between IL6 and stemness, which may include octamer 4 (OCT4)/NANOG expression and the clinical significance of IL6-IGFIR–mediated signaling as a potential therapeutic target for individualized adjuvant therapy and preventing early tumor relapse of HBV-HCCs.

**Translational Relevance**

This study shows that patients with hepatitis B virus (HBV)–related hepatocellular carcinoma (HBV-HCC) exhibit a high rate of concurrent expression of interleukin (IL)-6 in sera, and OCT4, NANOG, and insulin-like growth factor (IGF) I receptor (IGFIR) in primary tumors. We found the IL6–IGFIR signal mediates the expression of stemness-related properties that are associated with early recurrence and poor prognosis in patients with HCC. The IL6 stimulates the expression of IGFIR and autocrine IGF1STAT3 dependently in HBV-HCCs. Blockage of IGFIR signal by IGFIR-specific short hairpin RNA (shRNA) or IGFIR phosphorylation inhibitor PPP suppresses the IL6-induced stemness-related properties in vitro and in a xenograft mouse model. These results suggest that IL6–IGFIR signal is a potential target for individualized adjuvant therapy and preventing early tumor relapse of HBV-HCCs.

**Materials and Methods**

**Cell lines and HCC tissues**

The Hep3B (HBV+ HBsAg+ human HCC, HB-8064) and HepG2 (HBV+ human hepatoblastoma, HB-8065) cells were purchased from the American Type Culture Collection. The HuH7 (HBV+ human HCC) cells were obtained from the Japanese Collection of Research Bioresources. The HepG2.2.15 (HBV+ HBsAg+ human hepatoblastoma) cells were kindly provided by Dr. Jun-Jen Liu (Institute of Medical Biotechnology, Taipei Medical University, Taipei, Taiwan). The PLC5 (HBV+ HBsAg+ human HCC) cells were kindly provided by Dr. Ching-Tai Lin (Department of Medical Research, Chang Gung Memorial Hospital, Chiayi, Taiwan).

Cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Gibco-BRL) supplemented with 10% fetal bovine serum (FBS) and 3.7 g/L sodium bicarbonate. For the IGF1 treatment, cells were cultured in serum-free media with IGF1 (0–50 ng/mL; R&D Systems); for IL6 treatments, cells in serum-free media were treated with IL6 (50 ng/mL; Sigma-Aldrich) with or without the JAK/STAT3 inhibitor AG490 (Cayman Chemical) or S3I-201 (Merck Millipore). The solutions of 50 μmol/L AG490 and 1 μmol/L pircropodophyllin (PPP; Calbiochem), an IGFIR-phosphorylation-inhibitor, were prepared in dimethyl sulfoxide (DMSO).

Frozen HCC tissue samples were obtained from 191 patients (146 men) of ages 21 to 80 years with a mean age of 60.57 ± 11.24 years who had received curative hepatectomy between 2004 and 2012 at Chang Gung Memorial Hospital (Chiayi, Taiwan). Among the 191 patients, formalin-fixed, paraffin-embedded HCC tissue samples were available for 85 patients, and serum samples were available for 120 patients (Supplementary Fig. S1). The mean follow-up duration was 32.43 months (range, 1.2–106 months). Our study was approved by the Institutional Review Board of Chang Gung Medical Foundation. The study was conducted according to the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guideline (18) and a retrospectively analyzed, pathologic evaluation, and statistical analysis plan. The REMARK guideline was shown in Supplementary Table S1.

**RNA isolation and real-time quantitative reverse-transcription PCR**

Cell lines and frozen HCC tissues were subjected to total RNA isolation followed by real-time quantitative reverse-transcription PCR (qRT-PCR). Detailed descriptions are provided in the Supplementary Materials and Methods.

**Western blot analysis**

Proteins from the human HCC tissues and the cell lines were extracted with lysis buffer containing 0.1% SDS, 0.1% Nonidet P-40, 0.5% Sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, and 10 mmol/L Tris–HCl (pH 7.5) with a protease inhibitor cocktail (Roche Diagnostics). Detailed descriptions are provided in the Supplementary Materials and Methods. Detailed descriptions of the RNA isolation, real-time qRT-PCR analysis. Western blotting, immunohistochemical staining, dual luciferase assay, shRNA and lentivirus production, flow cytometry for side population cells, tumor sphere formation assay, xenograft tumor model, enzyme-linked immunosorbent assay (ELISA), and statistical analysis are provided in the Supplementary Materials and Methods.

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**Translational Relevance**

The purpose of this study was to investigate the interplay among IL6, IGFIR-mediated signaling, and OCT4/NANOG in primary tumors. We found that patients with HBV-HCC have a higher serum level of IL6, and the IL6 increases the expression of autocrine IGF1 and IGFIR, which then promote expression of the transcription factors OCT4 and NANOG in a STAT3-dependent manner. This study highlights the significance of IL6–IGFIR–mediated signaling as a potential therapeutic target for individualized adjuvant therapy for patients with HBV-related HCCs.
Results

Positive correlation between OCT4 and NANOG mRNA expression and IGFIR mRNA expression in human HCC tissues

To examine the associations between OCT4, NANOG, and IGFIR mRNA expression in human HCC, the tumor tissue (T) and adjacent peritumor tissue (PT) from 191 frozen tissue samples were analyzed using real-time qRT-PCR. As shown in Supplementary Fig. S2, mRNA levels of IGFIR were significantly correlated with mRNA levels of both OCT4 (R = 0.7281; P < 0.0001) and NANOG (R = 0.7144; P < 0.0001).

The expression levels of OCT4, NANOG, and IGFIR were also compared among patients with HCC infected with hepatitis C virus (HCV), HBV, HBV and HCV (BC), and those with neither HBV nor HCV (NBNC). The patients with HBV-HCC had significantly higher levels of OCT4, NANOG, and IGFIR mRNA compared with patients with HCV-HCC (Fig. 1A; P < 0.001 for all comparisons). When the 191 patients with HCC were divided into high (gene expression level of T/PT > 2) and low expression groups (gene expression level of T/PT < 2), the patients with HBV-HCC showed significantly higher rates of synchronous high expression of OCT4, NANOG, and IGFIR mRNA (Table 1; P = 0.038).

Association of OCT4, NANOG, and IGFIR expression with early tumor recurrence in human HCC

The clinical relevance of OCT4, NANOG, and IGFIR expression on the early tumor recurrence (within 2 years) of HCC was examined on the basis of the gene expression levels (either high [T/PT > 2] or low [T/PT < 2]) of these three genes in the 191 patients with HCC (Table 2, Fig. 1B, and Supplementary Fig. S3). Univariate analysis revealed that expression of OCT4 (P < 0.001), NANOG (P < 0.001), IGFIR (P < 0.001), and high expression of all three genes (P < 0.001) were significantly associated with early tumor recurrence (Table 2). HBV (P = 0.005), HCV (P = 0.029), tumor—node—metastasis (TNM) stage, microvascular/macrovascular invasion, satellite nodules, and tumor size (P = 0.001) were also significantly associated with early tumor recurrence (Table 2). Multivariate analysis also demonstrated that expression of OCT4 (P = 0.012), NANOG (P = 0.013), IGFIR (P < 0.001) and all three genes (P = 0.001) were significantly associated with early tumor recurrence.
recovery. The Kaplan–Meier curves of early tumor recurrence were significant for high versus low expression of the individual genes (OCT4, NANOG, or IGFIR; Fig. 1B; \( P < 0.0001 \)). The Kaplan–Meier curves of early tumor recurrence for concurrent high versus concurrent low expression of OCT4 and IGFIR or NANOG and IGFIR (Supplementary Fig. S3A; \( P < 0.0001 \)), for concurrent high expression of all three genes versus two genes or less (Supplementary Fig. S3B, left; \( P < 0.0001 \)) and for high expression of at least one of the three genes versus none of the three genes (Supplementary Fig. S3B, right; \( P < 0.0001 \)) were all significant. In addition, concurrent high expression of all three genes (OCT4, NANOG, and IGFIR) occurred 71% in hepatitis B–positive HCC (\( P = 0.014 \)), but only 35% in hepatitis C–positive HCC (\( P = 0.017 \); Supplementary Table S2). Concurrent high expression of all three genes was also correlated with the presence of microvascular invasion (\( P = 0.037 \) and satellite nodules (\( P = 0.008 \); Supplementary Table S2). There was also a significant difference between patients with HBV-HCC and patients with HCV-HCC in early tumor recurrence (Fig. 1B; \( P = 0.005 \)). The Kaplan–Meier curves of overall survival (OS; Supplementary Fig. S4) and the time-to-recurrence (Supplementary Fig. S5) for the gene expression were shown. The training/validation set analysis was further examined (Supplementary Fig. S6). Because of the lack of an external database of pluripotent transcriptional factor OCT4 and NANOG expression in HCC, we randomly divided our samples into two equal groups, the trained samples and the validation samples, to examine whether the associations between biologic measures and mortality were valid in our proposed model. As shown in Supplementary Fig. S6, there were no significant differences between the observed and predicted survival curves of IGFIR, OCT4, and NANOG (all \( P > 0.05 \) by the log-rank test). These results confirmed that our proposed model of mortality assessment through the use of biologic measures is valid.

### Table 1. Relationship between viral etiology and concurrent gene expression

<table>
<thead>
<tr>
<th>Number of high expression genes</th>
<th>HBV (( n = 73 ))</th>
<th>HCV (( n = 81 ))</th>
<th>BC (( n = 24 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OCT4, NANOG, and IGFIR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>69.2% (9/13)</td>
<td>46.6% (24/73)</td>
<td>74.1% (60/81)</td>
</tr>
<tr>
<td>1</td>
<td>23.1% (5/13)</td>
<td>16.4% (12/73)</td>
<td>13.6% (11/81)</td>
</tr>
<tr>
<td>2</td>
<td>0% (0/13)</td>
<td>11.0% (8/73)</td>
<td>12.5% (3/24)</td>
</tr>
<tr>
<td>3</td>
<td>7.7% (1/13)</td>
<td>26.0% (19/73)</td>
<td>4.2% (3/78)</td>
</tr>
<tr>
<td>0/1</td>
<td>12 (92.3%)</td>
<td>46 (63.6%)</td>
<td>71 (87.3%)</td>
</tr>
<tr>
<td>2/3</td>
<td>1 (7.7%)</td>
<td>27 (37.0%)</td>
<td>10 (12.3%)</td>
</tr>
<tr>
<td>( p )</td>
<td>0.038(^{b} )</td>
<td>0.268</td>
<td>0.446</td>
</tr>
</tbody>
</table>

NOTE: Expression genes include OCT4, NANOG, and IGFIR, \( n = 191 \).

\(^{a} \) \( \chi^2 \) test compared with the NBNC group.

\(^{b} \) \( P < 0.05 \).

### Table 2. Variables associated with early tumor recurrence after hepatocectomy for HCC (\( n = 191 \))

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis ( P )</th>
<th>( \hat{P} )</th>
<th>HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male vs. female)</td>
<td>0.571</td>
<td>NS</td>
<td>–</td>
</tr>
<tr>
<td>Age, y (&lt;55 vs. ≥55)</td>
<td>0.469</td>
<td>NS</td>
<td>–</td>
</tr>
<tr>
<td>HBV (yes vs. no)</td>
<td>0.005(^{a} )</td>
<td>0.216</td>
<td>1.49 (0.79–2.80)</td>
</tr>
<tr>
<td>HCV (yes vs. no)</td>
<td>0.029(^{a} )</td>
<td>0.984</td>
<td>0.99 (0.55–1.83)</td>
</tr>
<tr>
<td>Bilirubin, mg/dl (&lt;12 vs. ≥12)</td>
<td>0.232</td>
<td>NS</td>
<td>–</td>
</tr>
<tr>
<td>Albumin, g/dl (&lt;35 vs. ≥35)</td>
<td>0.078</td>
<td>0.276</td>
<td>1.34 (0.79–2.26)</td>
</tr>
<tr>
<td>ALT, U/L (&lt;35 vs. ≥35)</td>
<td>0.385</td>
<td>NS</td>
<td>–</td>
</tr>
<tr>
<td>PT, INR (&lt;12 vs. ≥12)</td>
<td>0.161</td>
<td>NS</td>
<td>–</td>
</tr>
<tr>
<td>AFP, ng/mL (&lt;400 vs. ≥400)</td>
<td>0.246</td>
<td>NS</td>
<td>–</td>
</tr>
<tr>
<td>ICG, retention rate (&lt;5% vs. ≥5%)</td>
<td>0.155</td>
<td>NS</td>
<td>–</td>
</tr>
<tr>
<td>TNM stage (T1/T2 vs. T3/T4)</td>
<td>&lt;0.001(^{a} )</td>
<td>0.803</td>
<td>1.10 (0.53–2.24)</td>
</tr>
<tr>
<td>Multiple tumors (yes vs. no)</td>
<td>0.749</td>
<td>NS</td>
<td>–</td>
</tr>
<tr>
<td>Child-Pugh class (A vs. B)</td>
<td>0.505</td>
<td>NS</td>
<td>–</td>
</tr>
<tr>
<td>Complete tumor capsule (yes vs. no)</td>
<td>0.362</td>
<td>NS</td>
<td>–</td>
</tr>
<tr>
<td>Microvascular invasion (yes vs. no)</td>
<td>&lt;0.001(^{a} )</td>
<td>0.005(^{a} )</td>
<td>2.22 (1.28–3.84)</td>
</tr>
<tr>
<td>Macrovascular invasion (yes vs. no)</td>
<td>&lt;0.001(^{a} )</td>
<td>0.226</td>
<td>1.97 (0.66–5.87)</td>
</tr>
<tr>
<td>Cutoff margin free (yes vs. no)</td>
<td>0.027(^{a} )</td>
<td>0.510</td>
<td>2.10 (0.69–2.30)</td>
</tr>
<tr>
<td>Tumor size, cm (&lt;3 vs. ≥3)</td>
<td>&lt;0.001(^{a} )</td>
<td>0.022</td>
<td>1.47 (0.79–2.74)</td>
</tr>
<tr>
<td>Satellite nodules (yes vs. no)</td>
<td>&lt;0.001(^{a} )</td>
<td>0.053</td>
<td>1.87 (0.99–3.52)</td>
</tr>
<tr>
<td>OCT4 expression (&lt;2X vs. ≥2X)</td>
<td>&lt;0.001(^{a} )</td>
<td>0.012(^{b} )</td>
<td>1.97 (1.36–2.33)</td>
</tr>
<tr>
<td>NANOG expression (&lt;2X vs. ≥2X)</td>
<td>&lt;0.001(^{a} )</td>
<td>0.013(^{b} )</td>
<td>1.98 (1.35–3.39)</td>
</tr>
<tr>
<td>IGFIR expression (&lt;2X vs. ≥2X)</td>
<td>&lt;0.001(^{a} )</td>
<td>0.001(^{b} )</td>
<td>2.86 (1.70–1.81)</td>
</tr>
<tr>
<td>High expression of all three genes of OCT4, NANOG, and IGFIR (&lt;2X vs. ≥2X)</td>
<td>&lt;0.001(^{a} )</td>
<td>0.001(^{b} )</td>
<td>2.65 (1.51–4.63)</td>
</tr>
</tbody>
</table>

Abbreviations: AFP, serum \( \alpha \)-fetoprotein; ALT, alanine aminotransferase; ICG, indocyanine green; PT, prothrombin time; INR, international normalized ratio; Tumor size, the largest one if multiple.

\(^{a} \) \( P < 0.05 \).

\(^{b} \) Time to early recurrence (less than 2 years).

Positive correlation between OCT4 and NANOG protein expression and IGFIR phosphorylation in human HCC tissues

OCT4 and NANOG protein expression levels and the activation of IGFIR by phosphorylation (p-IGFIR) in human HCC tumor tissues were examined by immunohistochemical staining (Fig. 1C). Figure 1C shows the immunohistochemical staining of OCT4, NANOG, and p-IGFIR in tumor and peri-tumor samples from one representative patient with HBV-HCC. The level of p-IGFIR positively correlated with the levels of OCT4 (\( R = 0.2377; P = 0.0285 \)) and NANOG (\( R = 0.2151; P = 0.0486 \)) in HCC tumor tissues (Fig. 1C; \( n = 85 \)). The expression of p-IGFIR/IGFB4, OCT4, and NANOG proteins in the tumor tissues was verified using Western blotting (Fig. 1D). These results highlight the association between IGFIR activation and expression of the pluripotent transcription factors OCT4 and NANOG in human HCC.

IGF1 and IGFIR regulate expression of OCT4 and NANOG in HBV-related human HCC cells

As the patient with HBV-HCC population displayed a greater proportion of patients with concurrent high expression of OCT4/NANOG and IGF1R (Table 1), and there is a positive correlation between the level of p-IGFIR (IGFIR activation) and OCT4/NANOG expression in HCC (Fig. 1C), we examined the effect of IGF1, a high-affinity ligand of IGFIR, on OCT4 and NANOG.
expression in HBV-HCC cells. We used human HCC cell lines that contain (Hep3B and HepG2.2.15) or do not contain (Huh7) the HBV genome. mRNA levels of OCT4 and NANOG were measured using qRT-PCR. As shown in Supplementary Fig. S7, IGFI treatment significantly increased OCT4 and/or NANOG mRNA expression in HBV+HBsAg+ Hep3B and HepG2.2.15 cells, but not in the HBV–HBsAg– Huh7 cell line. Because of the hepatoblastoma character of HepG2.2.15 cells, we used Hep3B and Huh7 as the differentiation cell model for further study. We found that IGFI had a dose-dependent effect on OCT4 and NANOG mRNA levels in Hep3B cells, although the effect on NANOG mRNA levels was not as pronounced. Huh7 showed a negative response to IGFI treatment (Fig. 2A). In addition, we performed experiments using shRNAs to knockdown the expression of the endogenous IGFI protein (shIGFIR#1 and shIGFIR#2) and found that shIGFI significantly suppressed the IGFI-induced OCT4 and NANOG expression in Hep3B (Fig. 2B) and HepG2.2.15 cells (Supplementary Fig. S8A). We also found that IGFI significantly increased the number of secondary tumor spheres of Hep3B and HepG2.2.15 cells in a serum-free medium, and that this effect was suppressed by either PPP (an IGFIR phosphorylation inhibitor) or shIGFIR#2 (Fig. 2C and Supplementary Figs. S8B and S8C). The IGFI effect on stemness-related properties was further supported by a side population assay, in which IGFI increased the percentage of Hep3B cells with the side population phenotype, and shIGFIR#2 significantly suppressed this effect of IGFI (Fig. 2D and Supplementary Fig. S9). These results suggest that IGFI and IGFIR may regulate the expression of OCT4 and/or NANOG in human HBV-HCC cells.

IL6 activates the expression of IGFI, IGFIR, and OCT4/NANOG in HBV-related human HCC cells

IL6 has been associated with the expression of genes that contribute to cancer stemness in HCC (7–9). We detected significantly higher levels of IL6 in the serum from patients with HBV-HCC compared with serum from patients with HCV-HCC (Fig. 3A, top). Patients with HCC who displayed high levels of OCT4 and NANOG expression also had significantly higher serum levels of IL6 (Fig. 3A, bottom), and the patients with high serum IL6 levels exhibited early tumor recurrence.
We found that IL6 induced the expression of both IGFIR and OCT4 in HBV⁻ HCC cells (HepG2 and Huh7; Fig. 3C, top). Additional studies demonstrated that IL6 dose-dependently increased the protein levels of secreted IGF1, IGFIR, OCT4, and NANOG in Hep3B cells (Fig. 3C, bottom). The effect of IL6 on IGF1/IGFIR and OCT4/NANOG expression was STAT3-dependent, because the JAK/STAT3 inhibitor AG490 (Fig. 3C, bottom) or S3I-201 (Supplementary Fig. S10) significantly suppressed the effects of IL6. Coomassie blue staining of the SDS-polyacrylamide gels shown in the bottom of Fig. 3C served as the loading control (Supplementary Fig. S11). Xenograft experiments also showed that IL6 strongly increased the expression of both IGF1 and OCT4, and to a lesser extent, NANOG, in Hep3B-derived tumors in vivo (n = 10). As shown in Fig. 3D, immunohistochemical staining of IL6-treated Hep3B tumors displayed higher levels of IGF1, OCT4, and NANOG protein than...
PBS-treated tissue samples. These results demonstrate that IL6 activates expression of IGFI, OCT4, and/or NANOG in HBV-HCC cells both in vitro and in vivo.

**IL6 increases stemness-related properties through IGFIR activation in HBV-related human HCC cells**

To examine whether IL6 regulates the stemness properties of HBV-HCC cells through the activation of IGFIR, RNA interference of endogenous IGFIR expression was performed. We found that the shIGFIRs suppressed the IL6-induced expression of OCT4 and NANOG in HBV+ Hep3B (Fig. 4A) and HepG2.2.15 cells (Supplementary Fig. S13A). IL6 treatment also increased the number of secondary tumor spheres (Fig. 4B, top and Supplementary Figs. S12 and S13B) and side population cells (Fig. 4B, bottom and Supplementary Fig. S14) of HBV-HCC cells; this effect of IL6 was suppressed by the addition of either PPP or shIGFIR. The xenograft experiments (Figs. 4C and Supplementary Fig. S15) provide additional support that the downstream effect of IL6 induces stemness via activation of IGFI/IGFIR signaling.

In the xenograft experiments using 1 x 10⁶ Hep3B cells for implantation, more tumors formed in IL6-treated mice than in PBS-treated mice (Supplementary Table S4). The risk of tumor formation significantly increased when ≥ 5 x 10⁶ cells were implanted (Supplementary Table S5; HR, 5.53; P < 0.001). In addition, immunohistochemical analysis showed strong OCT4 and NANOG expression in IL6-treated tumor cells (Fig. 4C; IL6 panel). Importantly, PPP markedly suppressed the IL6 effect on tumor growth (Supplementary Fig. S15; IL6 + PPP vs. IL6) as well.

**Figure 4.** IL6 increases stemness properties through IGFIR activation in human HBV-HCC cells. A, OCT4 and NANOG protein levels and the relative luciferase activity of the OCT4/NANOG promoter-luciferase shCtrl- or shIGFIR Hep3B cells with IL6 treatment (50 ng/mL). Student t test. B, top, the effect of IL6 and IGFIR on the formation of secondary spheres of Hep3B cells. Bottom, the percentage of Hep3B cells with the side population phenotype cultivated in medium only (Mock), IL6 (50 ng/mL), IL6 + shCtrl. or IL6 + shIGFIR#2. Student t test. C, immunohistochemical analysis of OCT4/NANOG expression following PBS, IL6, and IL6 plus PPP treatment (bar, 100 μm). D, serum levels of IL6 in patients with HCC stratified by expression level of all three genes (Low = T/PT < X, n = 51; high = T/PT > X, n = 23). By the Mann–Whitney U test. E, model of how IL6 increases the expression of pluripotent gene OCT4 and NANOG through IGFIR activation in human HBV-HCC cells. *, P < 0.05; **, P < 0.01; and ***, P < 0.001.
as OCT4/NANOG expression in Hep3B-derived tumors (Fig. 4C; panel of IL6–PPP vs. IL6). The strong correlation between IL6 and IGFIR/OCT4/NANOG was further supported by clinical observation. When compared with patients exhibiting low expression of all three genes, patients exhibiting high expression of the three genes had significantly higher serum levels of IL6 (Fig. 4D). These results suggest that IL6 increases pluripotency-related gene expression, particularly for OCT4, in HBV-HCC cells through the activation of IGFIR/IGFIR signaling pathway.

Discussion

The expression of pluripotency-related genes in tumors, such as OCT4 and NANOG, has been linked to poor prognosis and drug resistance (19, 20). However, the mechanisms regulating cancer stemness expression and tumor recurrence in HCC remain unclear. There is a definite causal relationship between inflammation and HCC, and the upregulation of certain inflammatory cytokines induced by HBV or HCV may contribute to hepatocarcinogenesis (21). However, the pathogenesis of HCC may vary among different etiologies. For example, a previous report showed that HCV infection can activate WNT signaling (13). The current work demonstrates that IL6 induces the IGFIR-mediated upregulation of OCT4 and NANOG expression, which is associated with early tumor recurrence in patients with HBV-HCC. This heterogeneity in the molecular pathogenesis and signaling pathways may contribute to the suboptimal efficacy of current therapies for HCC (22, 23). A better understanding of the different etiologies and their diverse mechanisms that drive tumorigenicity and/or tumor recurrence is crucial for the optimization of individualized therapy for HCC.

The mechanism for HBV-induced expression of pluripotency-related genes in cancer is still to be determined. Studies using a cell model of hepatoblastoma suggest that it occurs through activation of the HBx protein (24), while other studies suggest it occurs via hepatic progenitor cell activation (8, 25). How HBV induces pluripotency gene expression in differentiated HBV-infected hepatocytes still remains largely unknown. The current observation shows that patients with HCV-HCC and BC-HCC present lower expression levels of OCT4, NANOG, and IGFIR compared with patients with HBV-HCC (Fig. 1A). As HBV is frequently suppressed by IGFIR in dual HBV and HCV hepatitis (26), the fact that patients with BC-HCC expressed lower OCT4, NANOG, and IGFIR than did patients with HBV-HCC indirectly supports a role for HBV in OCT4, NANOG, and IGFIR expression, and emphasizes the etiologic differences that contribute to the prognosis of patients with HCC (12).

Niche environment might play a critical role in cell fate determination. Our current findings support an upstream role of HBV-induced inflammation in the development of stemness-related properties in HBV-HCC through IL6-induced IGFIR/IGFIR expression. The direct transactivation of IGFIR by IL6 was recently reported in prostate cancer (27). However, unlike prostate tumorigenesis, we found that IL6 did not directly transactivate the phosphorylation of IGFIR in Hep3B cells in either our IGFIR phosphorylation experiments (Supplementary Fig. S16) or in pull-down assays (data not shown). In contrast, our results showed that IL6 activates IGFIR through STAT3-dependent increases in IGF1 and IGFIR expression, and that IGFIR activation mediates the OCT4/NANOG expression in Hep3B cells. In support, studies of human embryonic stem cells and pluripotent mouse germ line stem cells demonstrated the role of IGFIR signaling in maintaining stemness (28–30). However, in contrast to the previous report that showed that NANOG-positive HCC cells may be IGFIR-mediated (31), we found that p-IGFIR levels were significantly correlated with OCT4 levels in HBV-HCC tumor tissues (Supplementary Fig. S17A); but, were even more significantly correlated with NANOG levels in HCV-HCC tumor tissues (Supplementary Fig. S17B). This result was further supported by our experiments using a xenograft tumor model that demonstrated the role of IGFIR activation as a dominant signal for IL6-induced stemness, particularly for OCT4, in HBV-HCC tumors (Fig. 4C). These results highlight the association of IGFIR activation with the expression of the different pluripotent transcription factors OCT4 and NANOG in etiologically different cancers.

Despite the large CD133-positive population in Hep3B cells, the tumor formation efficiency was relatively low in mice that did not receive treatment of IL6 (Supplementary Table S4) or IGF1 (data not shown). This result suggests a role for IL6 in stimulating HCC cell proliferation during the early stages of HCC. Furthermore, as IL6 is the principle cytokine in liver inflammation, the effects of IL6 may explain the lower incidence of HCC in patients with inactive carrier status compared with those with chronic active HBV hepatitis (3). When a larger number (1 × 10⁶) of Hep3B cells were implanted, the IL6 treatment increased cell proliferation only slightly while the expression of OCT4 increased significantly (Fig. 4C and Supplementary Fig. S15). These findings suggest that the IL6-induced pluripotency-related gene expression may have less effect on tumor proliferation with increasing tumor burden in late-stage HCC. The downstream IGFIR activation in IL6-induced OCT4/NANOG expression in HBV-HCC cells was also examined in the xenograft model using a low-dose PPP (1 mg/mL) treatment (16) to prevent cell apoptosis in the IL6-induced tumors. The low-dose PPP treatment not only reduced tumor size, but also markedly suppressed the IL6-induced expression of OCT4/NANOG in the tumors (Fig. 4C and Supplementary Fig. S15). These findings support the fact that activation of IGF/IGFIR mediated the downstream signaling in IL6-induced OCT4/NANOG expression in vivo.

The mechanisms regulating HCC recurrence are still largely unknown. The patients with HBV-HCC in our study displayed higher expression levels of pluripotency-related genes, especially of OCT4 and IGFIR, and tended to have early tumor recurrence (Fig. 1B) and worse OS (Supplementary Fig. S4) following hepatectomy. The significant impact of OCT4 and/or NANOG overexpression on tumor recurrence suggests that upregulated OCT4/NANOG confers the ability to micrometastasize. This association between pluripotency-related gene expression and micrometastasis of HBV-HCC is supported by the observation that NANOG-positive HCC cells expressed higher metastatic ability (31).

Although the disappointing preliminary results from clinical trials using IGFIR-specific monoclonal antibodies have raised doubts regarding the future of IGFIR inhibition in various cancers (32–34), several alternate approaches deserve attention. The blockage of IGFIR in HCC was shown to induce adaptive responses, such as upregulation of the epidermal growth factor receptor, thus emphasizing the rationale for cotargeting more than one signaling (35). Another approach is to facilitate HCC personalized therapy for treating IGFIR by incorporating biomarker data that have been under intense investigation (36). Although no optimal biomarkers have been identified for HCC thus far, our results suggested that IGFIR signaling inhibition
might work more effectively in the subset of HCCs associated with HBV infection.

In summary, HCC has a high rate of recurrence following treatment. Effective strategies for the prevention of HCC recurrence are needed to improve the prognosis of patients with HCC (37). In this study, we demonstrated that the inflammatory cytokine IL6 stimulates the autocrine IGF loop to activate IGFIR, and initiates OCT4 (and/or NANOG) expression in HBV-HCC cells (Fig. 4E). The IL6-induced expression of OCT4 and/or NANOG is primarily limited to patients with HBV-HCC, and is associated with early tumor recurrence. Our findings suggest that targeting both the IL6 and IGFIR signaling pathways would be the future strategies to improve individualized adjuvant therapy and prevent early tumor recurrence for patients with HBV-HCC.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: T.-S. Chang, Y.-C. Wu, T.-Y. Ling, Y.-H. Huang

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.-C. Wu, C.-C. Chi, K.-F. Lee, T.-H. Ting, J. Wang, S.Y. Tung, H.-N. Ho, Y.-H. Huang

Writing, review, and/or revision of the manuscript: T.-S. Chang, C.-C. Chi, T.-Y. Ling, Y.-H. Huang

References


Correction: Activation of IL6/IGFIR Confers Poor Prognosis of HBV-Related Hepatocellular Carcinoma through Induction of OCT4/NANOG Expression

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Reference


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Activation of IL6/IGFIR Confers Poor Prognosis of HBV-Related Hepatocellular Carcinoma through Induction of OCT4/NANOG Expression

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