Title:
Significant down-regulation of GHR expression as a new unfavorable prognostic factor in HCV-related hepatocellular carcinoma revealed.

Running title: Down-regulation of GHR expression in HCV-related HCC.

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Abbreviations

GH: Growth hormone; GHR: Growth hormone receptor; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; DCV: daclatasvir; LC: liver cirrhosis; TLCN: Taiwan Liver Cancer Network; ANT: Adjacent non-tumor tissues; RT-qPCR: Real-time quantitative PCR; JAK2: Janus kinase 2; STAT5: Signal transducer
and activator of transcription 5; IGF1: Insulin-like growth factor 1; AFP: Alpha-fetoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; SD: Standard deviation; CI: Confidence interval; AJCC: American Joint Committee on Cancer

Abstract

Background/Aims: Growth hormone (GH) is the main regulator of somatic growth, metabolism, and gender dimorphism in the liver. GH receptor (GHR) signaling in cancer is derived from a large body of evidence, although the GHR signaling pathway involved in the prognosis of hepatocellular carcinoma (HCC), especially in hepatitis C virus (HCV)-related HCC, remains unclear. We aimed to explore the expression of GHR and analyze its association with clinicopathologic features and prognosis of HCV-HCC patients.

Methods: The expression of GHR mRNA was investigated by quantitative real-time PCR (RT-qPCR) in paired tumor and adjacent non-tumorous (ANT) liver tissues of 200 HCV-HCC cases. Western blotting and immunofluorescence assays using the HCV-infected Huh7.5.1 cell model was performed.

Results: GHR mRNA was significantly lower in HCV-HCC tissues than corresponding ANT. GHR mRNA and protein levels also decreased in the HCV-infected Huh7.5.1 cell model. Notably, lower GHR expression was associated with age >60 years (p = 0.0111) and the worse clinicopathologic characteristics including AFP >100 ng/ml (p = 0.0403), cirrhosis (p = 0.0075), vascular invasion (p = 0.0052), pathological stage II-IV (p = 0.0002) and Albumin ≤ 4.0 g/dL (p = 0.0055), which were linked with poorer prognosis of HCC. Most importantly, the higher incidence of recurrence and poor survival rates in patients with a low ratio of tumor/ANT GHR (≤ 0.1) was observed, indicating that lower expression levels of GHR had great risk for HCV-HCC progression.

Conclusion: Our study demonstrates a significant down-regulation of GHR expression as a new unfavorable independent prognostic factor in HCV-HCC.
Keywords:
Growth hormone receptor, Hepatocellular carcinoma, Hepatitis C virus, Recurrence, Prognostic factor

Study Highlights:
- The GHR expression level was significantly lower in human HCV-related HCC tumor tissues than corresponding adjacent non-tumorous (ANT) liver tissues.
- A lower tumor/ANT GHR ratio of < 0.1 was associated with poor prognosis of HCV-HCC patients after tumor resection, in terms of higher incidence of HCC recurrence and poorer survival.
- A significant down-regulation of GHR expression as a new unfavorable independent prognostic factor in HCV-HCC.
Introduction

Around 2-3% of the world population is chronically infected with hepatitis C virus (HCV), which is one of the leading causes of end-stage liver diseases such as liver cirrhosis (LC), hepatic decompensation and hepatocellular carcinoma (HCC) \(^1\),\(^2\). HCC is one of the most prevalent cancers in human beings, ranking 5th worldwide and 2nd in Taiwan \(^3\), and serves as the third leading cause of cancer mortality \(^4\).

HCV-induced HCC development is a multi-step process that could take 20–40 years, and each step in the process could be a target for the prevention of HCC \(^5\). Viral, host, and environmental factors have been associated with increased risk of HCV-related HCC, including HCV genotypes, the severity of liver fibrosis, genetics variants, and serum levels of MHC Class I Chain-related A, and alcohol intake \(^6\)-\(^10\). Successful antiviral therapy has been reported to greatly reduce the incidence of HCC \(^7\); however, the risk of HCC remains among patients even after eradication of HCV, especially the elderly with advanced liver diseases, high baseline gamma-glutamyl transferase (rGT), and diabetes \(^11\)-\(^13\). Furthermore, the development of HCC has been observed in mice expressing HCV transgenes in the absence of appreciable hepatic inflammation and fibrosis, suggesting that HCV infection is likely to have direct and unique cancer-promoting effects \(^14\). The first choice in the management of HCC in the early stage is surgical resection \(^15\); nevertheless, the 5-year recurrence rate remains as high as 60% even after curative resection \(^16\). Factors associated with HCC recurrence include tumor size \(^17\), fibrosis \(^18\), vascular invasion \(^19\), satellite lesions \(^20\), serum AFP, etiologies \(^21\) and serum albumin levels \(^22\). Understanding the factors and mechanisms associated with HCV-related HCC development and recurrence could provide potential targets and promising strategies to improve cancer survival \(^23\).
Growth hormone (GH) is the main regulator of somatic growth, metabolism, and gender dimorphism in the liver \(^{24}\). The GH receptor (GHR) was first identified and characterized in the liver \(^{25}\), a predominant target organ for GH \(^{26}\). GH binds its cognate receptor GHR, activating Janus kinase 2 (JAK2), which in turn phosphorylates the signal transducer and activator of transcription 5 (STAT5) and facilitates the expression of several GH downstream genes, including insulin-like growth factor 1 (IGF1), an important effector of GH action \(^{27,28}\). The effect of the GH/IGF-1 system on cancer progression has recently been the focus of much interest. GH/IGF-1 axis dysregulation enhances the synergistic effect of GH and IGF-1 on the promotion of uncontrolled cell proliferation, cell movement, and angiogenesis, as well as the increase of neoplasia risk \(^{29}\). Importantly, disruption of GH-JAK2-STAT5-IGF1 signaling is associated with hepatic metabolic changes and pathogenesis of fatty liver, fibrosis, LC, and HCC \(^{24}\), while STAT5 activation is correlated with the progression of human HCC \(^{30}\). In apparent contrast, the loss of STAT5 in mice causes liver steatosis and fibrosis and promotes chemically-induced HCC, by altering the expression of cell cycle pathway regulators \(^{31}\). A recent animal study showed that hepatic GH signaling is crucial for the maintenance of lipid homeostasis and that the impairment of this signaling causes severe metabolic liver disease predisposing to HCC \(^{32}\). In humans, low levels of circulating IGF1 and high levels of GH, indicative of GH resistance, were commonly found in patients with liver fibrosis and cirrhosis \(^{33}\) and has been implicated in the development of HCC \(^{34}\). However, whether the GH-STAT5-IGF1 signaling pathway is involved in the prognosis of HCV-related HCC remains unclear.

In the present study, we firstly identified that GHR was highly down-regulated in human HCV-HCC tumor tissues as compared to adjacent non-tumor tissues (ANT). Then, the effects of HCV infection on the GH-STAT5-IGF1 signaling pathway were evaluated in HCV infectious clones. Finally, the prognostic role of the GH-STAT5-IGF1 signaling pathway in a cohort of HCV-HCC post curative tumor resection was validated by determining the expression levels of GHR and its downstream genes in HCC tumor and ANT tissues. We innovatively revealed that there are interplays between virus and host through the dysregulation of GHR and its downstream genes that were correlated to the poor prognosis and low survival rate of HCV-related HCC after surgical resection.
Materials and Methods

Study population

For empirical validations, HCC tumor tissues with paired ANT liver tissues and associated clinical information of 200 chronic hepatitis C patients who underwent surgical resection of HCC were provided by the Taiwan Liver Cancer Network (TLCN). Tumor specimens and paired ANT liver tissue were obtained immediately after surgical resection. This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital and the TLCN User Committee.

Cell culture and Generation of HCVcc stocks and experimental infection.

In this study, Huh7.5.1 cells were obtained from ATCC. Huh7.5.1 cells were maintained in DMEM (Gibco) with 10% fetal bovine serum (Gibco). The cultural medium also contained penicillin (100 units/mL), streptomycin (100 μg/mL), L-glutamine (2 mmol/L), and sodium pyruvate (1 mmol/L) (Gibco) at 37°C in a humidified incubator containing 5% CO₂. For a generation of HCVcc stocks, Huh7.5.1 cells were transfected with Chimeric J6/JFH genome genotype 2a HCV cDNA from Dr. Lan Keng-Hsin. Transfected cells were incubated for six days, and virus (HCVcc)-containing supernatants were collected. For infection, Huh7.5.1 cells were seeded at a density of 1x10⁴ or 1x10⁵ in 12- or 6- well dishes and infected with HCVcc at a multiplicity of infection (m.o.i.) of 0.5, respectively. At 24 hours post-infection, the HCV-infected Huh7.5.1 cells were treated with 5 pM DCV for three days. The supernatant was replaced with fresh media 6 hours post-infection. At 2, 3, and four days post-infection, cells were harvested for RNA isolation, immunostaining, and Western blotting analysis.

RNA isolation and cDNA synthesis

Total RNA extraction was performed using Quick-RNA™ MiniPrep Kits for cells according to the manufacturer’s instructions (Zymo Research, USA). Total RNA extracted from HCV-related HCC tumors and ANT tissues were obtained from the tissue bank of TLCN. The cDNA was synthesized from 1 μg of
total RNA incubated in a 20μl volume reaction using the Superscript III kit for RT-PCR (Invitrogen, Life Technologies, USA) with random primers.

**Quantitative real-time reverse transcription-PCR**

The relative abundances of GHR, IGF-I, CDKN1A, 5'UTR, and NS5B mRNA in the HCV-infected Huh 7.5.1 cells and the liver bio-samples were detected using quantitative real-time reverse transcription-PCR (RT-qPCR). The real-time PCRs were performed using the ABI PRISM 7900 system (Applied Biosystems) and SYBR GreenER™ qPCR SuperMix (Invitrogen, Life Technologies, USA) with cycling conditions of 20 s at 95 °C and 40 cycles of 1 s at 95 °C and 20 s at 60 °C. The ΔCt (threshold cycle) method was used to calculate the relative abundance of mRNA and expressed as a tumor ΔCt / non-tumor ΔCt (T/NT) ratio in the liver bio-samples. The changes in gene expression were analyzed by the ΔΔCt method, using GAPDH as an endogenous control. All samples were analyzed in triplicate. All primers are listed in Supplementary Table 4.

**Immunofluorescence Staining and Confocal Microscopy**

For immunofluorescent labeling, the Huh 7.5.1 cells were grown on glass coverslips at a density of 1x10^5 cells in 12-well dishes overnight. After post-infection, cell cultures were rinsed several times with PBS and fixed in 4% paraformaldehyde for 5 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min. Fixed cells were rinsed in PBS. Non-specific binding was blocked with 5% normal goat serum (NGS) / 1% bovine serum albumin (BSA) in PBS pH 7.4 for at least 30 min at 37°C. After a brief wash, the cells were incubated for 45 min at 37°C with the primary antibodies [e.g. anti-HCV core protein (Millipore) and anti-GHR (Abcam)]. After extensive washes with PBS, the cultures were then incubated with the appropriate secondary antibody conjugated to either Alexa 488 or Alexa 568 (Molecular Probes, USA) for 45 min at 37°C. Finally, the slides were incubated for 5 min with DAPI (Molecular Probes, USA) before mounting with Prolong Gold antifade reagent (Molecular Probes, USA). Confocal images were obtained using a ZEISS LSM700 microscope, controlled by ZEN software (Carl Zeiss Group, Hartford, CT, USA). All images were imported into Adobe Photoshop v7.0 for contrast manipulation.
Western blotting

The cellular lysate was prepared using RIPA buffer, and the protein content was determined by a Bio-Rad Protein Assay system. Proteins were separated on 10% or 12% SDS-PAGE and transferred onto PVDF membrane. Then the membrane was incubated with GHR (Abcam), anti-phospho Stat5 and anti-total Stat5, (Cell Signalling), anti-HCV core protein (Millipore), or anti-β-actin (Sigma-Aldrich). The secondary antibody used was goat anti-mouse or anti-rabbit IgG conjugated to HRP, and the ECL reagents (Amersham) were used for immunodetection. The chemiluminescent signal was captured by an Image Quant™ LAS 4000 mini system (GE Healthcare Life Sciences).

Statistical analysis

All categorical and continuous variables were analyzed by $\chi^2$ test, and group means (presented as the mean ± standard deviation) were compared using analysis of variance and Student’s $t$-test. For continuous variables with outliers, nonparametric Mann-Whitney tests were used. The area under the curve by using receiver-operating characteristics (AUROC) was performed and the cut-off point tumor/ANT GHR ratio was determined to best distinguish the risk of HCC recurrence of HCV-HCC patients after surgical resection. Kaplan–Meier analysis and the Log-rank test were performed by comparing the differences in the cumulative incidence of HCC among patients with tumor/ANT GHR ratio $> 0.1$ and $\leq 0.1$. The risk factors independently associated with HCC development were evaluated by using Cox regression analysis. The statistical analyses were performed using the JMP software (version 9). All statistical analyses were based on two-sided hypothesis tests with a significance level of $p<0.05$.

Results

GHR expression was down-regulated in HCV-related HCC.

To examine what differential expressions of host genes between tumor and ANT tissues in patients with HCV-related HCC, we employed microarray analysis to reveal gene profiles from three HCV-related HCC
liver tissue samples. Our exploratory gene array data showed that there were 69 up-regulated and 165 down-regulated genes with at least a two-fold change (Supplementary Table 1, Table 2 and Supplementary Fig. 1). GHR was one of the down-regulated genes in a subset of these samples. As mentioned above, GH-STAT5-IGF1 signaling was associated with the pathogenesis of liver diseases and HCC. However, the effects of HCV on GHR expression and the molecular mechanisms in HCC have not been elucidated. To further validate these findings, we first detected the expression level of GHR in 200 paired HCV-related HCC tissue specimens from the Taiwan Liver Cancer Network (TLCN) by real-time quantitative PCR (RT-qPCR). We observed that GHR expression level decreased in 89% (178 of 200 cases) and increased in 11% of HCV-related HCC cases (22 of 200 cases). The biochemical and clinicopathological characteristics of the study population are outlined in Table 1. The relative expression of GHR mRNA in HCV-related HCC tumor tissues was significantly lower than that in ANT (2.557 ± 5.341 vs. 15.780 ± 20.591, p < 0.0001) (Supplementary Fig. 2A and Supplementary Table 3). We further examined the IGF1 and CDKN1A expression levels, which were downstream genes of the GH-STAT5-IGF1 signaling pathway. Our data showed that the expression levels of IGF1 and CDKN1A also decreased in HCV-related HCC tumorous tissues compared to adjacent non-tumorous tissue (Supplementary Fig. 2B and 2C, Supplementary Table 3). These findings revealed that GHR and its downstream genes IGF1 and CDKN1A were significantly down-regulated in HCV-related HCC tumorous tissues.

**Down-regulation of GHR in the HCV-infected Huh7.5.1 cell model.**

To examine whether HCV infection regulates GHR expression, we performed the cell experiments using the HCV (J6/JFH) infected Huh7.5.1 cell model. Our data showed that the production of infectious HCV gradually increased with time. This simulated the gradual increase of infected cells over time under physiological conditions. The infected cells appeared grossly to cluster at Day 2 and Day 3 and then spread out on Day 4 (Fig. 1A). GHR expressions were measured by RT-qPCR in HCV-infected cells. The expression of HCV 5’UTR and NS5B increased concomitantly with decreased expression of GHR overtime during HCV infection (Fig. 1B). Moreover, we used an antibody to detect the expression level of GHR in HCV-infected cells by immunostaining. The GHR expression was evenly scattered in the cytoplasm and
membrane in non-HCV-infected cells, but it significantly decreased in HCV-infected cells (Fig. 1C). Our data suggested that GHR levels were correlated negatively to HCV viral load in the HCV-infected cells.

The HCV effect on the GH-STAT5 signaling pathway in the HCV-infected Huh7.5.1 cell model.

The growth hormone binds its cognate receptor GHR, which results in the activation of the GH-STAT5 signaling pathway. We further investigated whether GHR expression and activation of the GH-STAT5 signaling pathway were regulated by the HCV infection. HCV-infected cells were treated with daclatasvir (DCV), a potent direct-acting antiviral agent to inhibit HCV replication. We demonstrated that HCV replication was robustly inhibited after 3-day daclatasvir treatment in HCV-infected cells (Fig. 2A and 2B) concomitantly with the moderate restoration of GHR expression (Fig. 2B and 2C, lane 2 compared to lane 3). This finding suggested that GHR is negatively regulated by HCV infection and able to be restored by inhibiting HCV replication. We further examined the status of STAT5 involved in the GH-STAT5 signaling pathway. Our data showed that the phospho-STAT5 (activated form) was moderately reduced in HCV-infected cells (Fig. 2C, lane 2 compared to lanes 1 and 3). Furthermore, we used growth hormones to stimulate the GH-STAT5 signaling pathway during HCV infection. The phospho-STAT5 (activated form) significantly increased in control and daclatasvir treatment groups, but not in the HCV-infected group upon growth hormone administration (Fig. 2C, lanes 4 and 6 compared to lane 5). Taken together, these findings implicate that GHR was negatively regulated by HCV infection and affected the GH-STAT5 signaling pathway in the HCV-infected Huh7.5.1 cell model.

Correlations between GHR expression and clinical characteristics of HCV-HCC patients

The correlation between GHR and the clinicopathological characteristics in HCV-related HCC patients was further evaluated by using the integer-fittest value with the AUROC curve for tumor/ANT GHR ratio to predict HCC recurrence. Our analysis excluded 7 out of the 200 HCC who developed recurrence when receiving OP. Therefore, during the follow-up period, all samples are confirmed post-operative for recurrence. We observed that tumor/ANT GHR ratio ≤ 0.1 was the best cutoff value for predicting HCC recurrence after surgical resection with sensitivity, specificity and AUROC of 49.6%, 79.0%, and 0.657
respectively. We further stratified patients into two groups dependent on the tumor/ANT GHR ratio > 0.1 or ≤ 0.1 to evaluate the role of tumor/ANT GHR expression ratio in the clinical characteristics of HCV-HCC patients. Compared to HCV-HCC patients with higher tumor/ANT GHR ratio (> 0.1), those with lower tumor/ANT GHR ratio had significantly higher proportion of patients with age > 60 years (81.2% vs. 62.6%, \(p = 0.0051\)), with AFP >100 ng/mL (47.1% vs. 28.1%, \(p = 0.0047\)), with vascular invasion (60.7% vs. 38.3%, \(p = 0.0023\)), with advanced pathological stage (II-IV) of HCC (68.2% vs. 52.2%, \(p = 0.0167\)), and higher risk of HCC recurrence (85% vs. 59.8%, \(p = 0.0002\)). However, lower tumor/ANT GHR ratio did not correlate to gender, tumor size, HCV genotype, history of HCV treatment, viral loads, cirrhosis status, levels of AST, ALT, albumin and BMI (all \(p > 0.05\), Table 2). We also observed that the tumor/ANT ratio of IGF and CDKN1A was significantly lower among patients with lower tumor/ANT GHR ratio, compared to those of higher tumor/ANT GHR ratio (both \(p < 0.0001\), Table 2).

Relationship between the tumor/ANT GHR expression ratio and prognosis of HCV-HCC patients after surgical resection

With a median follow-up period of 65.0 months (range: 1–119 months, total 231.6 person-years), after surgical resection, 128 (64.0%) of 200 HCV-HCC patients experienced HCC recurrence and 86 (43%) patients had expired, with an annual incidence of HCC recurrence at 1.81% and annual death at 5.15%. The 1-, 3-, and 5-year cumulative HCC recurrent rates were 29.2%, 53.8%, and 64.3% respectively. Using Kaplan-Meier and log-rank survival tests, we demonstrated that the 1-, 3-, and 5-year cumulative rates of HCC recurrence were 40.4%, 73.9%, and 82.1% respectively, for patients with tumor/ANT GHR ratio ≤ 0.1, compared to 21.1%, 39.4%, and 51.7% respectively, for patients with tumor/ANT GHR ratio > 0.1 (HR 2.25, 95% CI 1.59–3.22, \(p < 0.001\), Fig. 3A). For overall survival, the 1-, 3-, and 5-yr cumulative rates of survival after HCC resection in HCV-HCC patients with tumor/ANT GHR ratio ≤ 0.1 were 88.1%, 70.2%, and 55.3% respectively, which were significantly lower than 93.8%, 85.0%, and 75.0% respectively, among those with tumor/ANT GHR ratio > 0.1 (HR 2.57, 95% CI 1.67–4.02, \(p < 0.001\), Fig. 3B). We further evaluate the predictive value of GHR in HCC recurrence and overall survival among specific subgroups of HCV-related HCC patients. Among patients with age > 60 years, those with a lower tumor/ANT GHR
expression ratio (≤ 0.1) remained at significantly higher risk of HCC recurrence ($p = 0.0019$) and mortality ($p = 0.004$) (Fig. 3C and 3D). Similarly, among those with advanced pathological stages II-IV, patients with a lower tumor/ANT GHR expression ratio (≤ 0.1) still had significantly higher rate of HCC recurrence ($p = 0.022$) and shorter OS ($p = 0.0035$) (Fig. 3E and 3F).

**Factors associated with HCC recurrence and overall survival in HCV-related HCC patients’ surgical resection**

*Univariate analysis*

Univariate analysis by Cox Hazard model showed that lower tumor/ANT GHR ratio (≤ 0.1) ($p = 0.0014$), presence of cirrhosis ($p = 0.0167$), and advanced pathological stage II-IV ($p = 0.0049$) were significantly associated with shorter time to HCC recurrence after surgical resection for HCV-HCC (Table 3). For overall survival, lower tumor/ANT GHR ratio (≤ 0.1) ($p < 0.0001$), age >60 years ($p = 0.0075$), vascular invasion ($p = 0.0061$), and advanced pathological stage II-IV ($p = 0.0001$) were significantly associated with worse overall survival in HCV-HCC patients after HCC resection (Table 4).

*Multivariate analysis*

Subsequently, multivariate Cox’s proportional hazard regression analysis was performed on three or four prognostic factors that showed statistical significance for HCC recurrence and/or mortality in univariate analysis ($p < 0.05$). Lower tumor/ANT GHR ratio (≤ 0.1) (HR, 2.40; 95% CI, 1.26–4.54; $p = 0.0075$), presence of cirrhosis (HR, 2.05; 95% CI, 1.11–3.78; $p = 0.0214$), and advanced pathological stage II-IV (HR, 2.14; 95% CI, 1.15–3.96; $p = 0.0153$) were independent risk factors for HCC recurrence after surgical resection for HCV-HCC patients (Table 3); while lower tumor/ANT GHR ratio (≤ 0.1) (HR, 3.14; 95% CI, 1.68–5.98; $p = 0.0004$), age >60 years (HR, 2.12; 95% CI, 1.04–4.30; $p = 0.0376$), and advanced pathological stage II-IV (HR, 5.52; 95% CI, 1.93–15.74; $p = 0.0014$) were independent predictors of mortality (Table 4). Our data suggest that a lower tumor/ANT GHR ratio (≤ 0.1) was an important risk factor associated with HCC recurrence and mortality in HCV-related HCC patients after surgical resection.
Discussion

In the current study, we firstly validated and revealed that the GHR expression level was significantly lower in human HCV-related HCC tumor tissues than corresponding ANT liver tissues. We also observed that HCV infection down-regulated the mRNA and protein levels of GHR in the cell model. Notably, lower tumor/ANT GHR expression ratio was associated with old age (>60 years) and worse clinicopathologic characteristics of HCC, including high AFP levels, vascular invasion, and advanced pathological stage of HCC, which were linked with poorer prognosis in HCC. Most importantly, we found that a lower tumor/ANT GHR ratio of ≤ 0.1 was associated with poor prognosis of HCV-HCC patients after tumor resection, in terms of higher incidence of HCC recurrence and poorer survival.

Several studies have reported that both expressions of GHR mRNA and protein were elevated in human cancers 35-38. Conway-Campbell et al. have reported nuclear localization of GHR induces dysregulated cell cycle progression and tumorigenesis 36. Basu et al. also demonstrated that siRNA-mediated GHR knock-down attenuated tumor proliferation, migration, and invasion in human melanoma cells 37. All these studies demonstrated that GH binds to aberrantly expressed GHR and activates the JAK2 kinase, driving EMT and promoting tumor progression. García-Caballero et al. revealed that GHR shows high expression in HCC than the normal liver by immunohistochemistry staining 39. On the contrary, a lower GHR level has led to the development of GH resistance and is associated with more advanced liver disease and fibrosis progression 40. To our knowledge, the role of GHR in the development of HCC has never been explored in the HCV cohort.

In our study, we showed that GHR and its downstream genes, IGF1 and CDKN1A, were significantly down-regulated in HCV-HCC tumorous tissues. More importantly, the suppression of GHR was highly associated with HCC recurrence and poorer survival rates in HCV-related HCC patients. The debate may be due to various reasons. Firstly, the HCV infection often has several effects on inflammation, oxidative stress, insulin resistance, steatosis, and progressive fibrosis in the liver 41. At the same time, HCV also dysregulates the cell cycle causing cell proliferation 42-44. The multiple incidents induced by HCV-infection probably modulate GHR expression. Secondly, human GHR is expressed abundantly in liver tissue compared to other tissues. The down-regulation of GHR might lead to interfering with the GH signaling pathway in the
liver. Besides, our data show CDKN1A, one of the key cell cycle inhibitors, declined in HCV-related HCC specimens. This result suggested that the down-regulation of CDKN1A might promote cell cycle progression in HCV-HCC. The data is consistent with a previous report that GH-mediated STAT5 negatively regulated cell proliferation through the activation of CDKN2B and CDKN1A expression. Finally, other signaling pathways such as the Ras/extracellular signal-regulated kinase (ERK) and PI3-kinase/AKT are also activated by GHR. Dysregulation of the GH–IGF-1 axis could promote uncontrolled cell proliferation, angiogenesis, and suppress apoptosis to increase the risk of neoplasia. Our findings indicated the potential significance of the GH-STAT5 signaling pathway in HCV-mediated hepatocarcinogenesis.

In the current study, we found that a lower tumor/ANT GHR expression ratio was associated with worse clinicopathologic characteristics including older age, high AFP levels, cirrhosis, vascular invasion, advanced stage, and lower albumin level, which are related to the poor prognosis in HCC, suggesting that GHR might be closely related to the progress of hepatocarcinogenesis. Our data indicated that the down-regulation of GHR might lead to cell cycle defects and further promote the progression of HCV-HCC.

Previous studies have shown that old age was not only an unfavorable factor for HCV outcomes. This study confirmed that old age is a negative predictor of 5-year recurrence-free outcome, and overall survival of patients with low GHR expression ≤60 years was significantly higher than those > 60 years of age. This indicated that age is important for post-treatment recurrence and patient survival results. Nevertheless, we observed that lower tumor/ANT GHR ratio still played an important role in poorer long-term outcomes among older HCV-HCC patients after surgical resection. Similar results were also observed where a lower tumor/ANT GHR ratio remained a negative predictor of long-term outcomes among HCV patients who had advanced HCC pathological stage after surgical resection.

Many studies have shown that the size and number of tumors are important for post-treatment recurrence and patient survival outcomes. However, in our cohort, tumor size and number had no significant effect on prognosis by multivariate analysis. This indicated that the prognosis of HCV-HCC patients was related to multiple factors, and comprehensive information should be evaluated. Given that GHR was strikingly down-regulated in HCV-HCC tissues, which was closely associated with age and the
advanced pathological stage (II-IV), we suggest that GHR might serve as a novel biomarker to predict prognosis outcome for HCV-HCC patients. Taken together, our data suggest that GHR is a sensitive clinical parameter predicting the HCC recurrence and survival of HCV-HCC patients and could serve as a useful prognostic molecular marker for various HCV-HCC subgroups.

There are some limitations in this study. Firstly, the design is retrospective. To confirm our findings, it is necessary to conduct a prospective study with a longer follow-up period. Secondly, our research showed that the effect of HCV infection would lead to the down-regulation of GHR, causing a decrease in IGF-1. It should be noted that IGF-1 also has negative feedback in regulating GH secretion, and patients with low IGF-1 levels might be the cause of excessive GH secretion. So far, no good animal model of HCV infection has been established, and it is difficult to measure GH levels in serum for verification. Thirdly, it is very interesting to compare the expression of GHR and its downstream pathway among CHC (non-LC) and CHC-LC and CHC-HCC. But the HCV-related HCC tumors and ANT tissues were obtained from the patients with HCC resection. We cannot obtain liver tissues from CHC patients as well as CHC-LC patients in this study. Hence, we couldn’t check the expressions of GHR and its downstream pathway between CHC (non-LC) and CHC-LC. Finally, the relationship between HCV-related down-regulation of GHR expression and HCV-HCC occurrence needs to be further studied.

Conclusions

In this study, we revealed GHR is down-regulated in human HCV-HCC tissues and HCV-infected Huh7.5.1 cell models, which might contribute to HCV-HCC recurrence and disease progression. A lower tumor/ANT GHR expression ratio was associated with a higher risk of HCC recurrence and mortality among HCV-HCC patients after surgical resection, indicating that GHR might be a promising biomarker and a potential therapeutic target for the patients with HCV-HCC.
# TABLES

Table 1. Clinicopathological information of HCV-related HCC patients obtained from the TLCN

<table>
<thead>
<tr>
<th>Clinical character</th>
<th>Variable</th>
<th>No. of Patients (n = 200)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, n (%)</td>
<td>Male</td>
<td>128 (64.0%)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>72 (36.0%)</td>
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<tr>
<td>Age, n (%)</td>
<td>&gt; 60 years</td>
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<tr>
<td></td>
<td>≤60 years</td>
<td>59 (29.5%)</td>
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<td>Tumor size, n (%)</td>
<td>&gt; 5 cm</td>
<td>52 (26.0%)</td>
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<td></td>
<td>≤5 cm</td>
<td>148 (74.0%)</td>
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<tr>
<td>AFP, (ng /ml, mean ± SD)</td>
<td>&gt; 100 ng /ml</td>
<td>72 (36.2%)</td>
</tr>
<tr>
<td></td>
<td>≤100 ng /ml</td>
<td>127 (63.8%)</td>
</tr>
<tr>
<td>Clinical Character Variable</td>
<td>Tumor/ANT GHR &gt; 0.1, (n=115)</td>
<td>Tumor/ANT GHR ≤ 0.1, (n=85)</td>
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<tr>
<td>Gender, Male n (%)</td>
<td>74 (64.3%)</td>
<td>54 (63.5%)</td>
</tr>
<tr>
<td>Female n (%)</td>
<td>41 (35.7%)</td>
<td>31 (36.5%)</td>
</tr>
<tr>
<td>Age, &gt; 60 years, n (%)</td>
<td>72 (62.6%)</td>
<td>69 (81.2%)</td>
</tr>
<tr>
<td>≤60 years, n (%)</td>
<td>43 (37.4%)</td>
<td>16 (18.8%)</td>
</tr>
<tr>
<td>Tumor size, &gt; 5 cm , n (%)</td>
<td>82 (71.3%)</td>
<td>66 (77.6%)</td>
</tr>
<tr>
<td>≤5 cm</td>
<td>33 (28.7%)</td>
<td>19 (22.4%)</td>
</tr>
<tr>
<td>AFP, &gt; 100 ng /ml , n (%)</td>
<td>32 (28.1%)</td>
<td>40 (47.1%)</td>
</tr>
</tbody>
</table>

HCV, hepatitis C virus; AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma; AST, aspartate aminotransferase; ALT, alanine aminotransferase; SD, standard deviation; Pathological stage according to AJCC and UICC, 7th ed.49

Table 2. Clinical characteristics of HCV-HCC patients according to the ratio of GHR expression (Tumor/ANT)
<table>
<thead>
<tr>
<th>Clinical character</th>
<th>Variable</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>Tumor/ANT GHR ratio, (≤ 0.1 vs &gt; 0.1)</td>
<td>2.68(1.46-5.07)</td>
<td>0.0014*</td>
<td>2.40(1.26-4.54)</td>
</tr>
<tr>
<td>Gender, (Male vs Female)</td>
<td>0.75(0.41-1.39)</td>
<td>0.3708</td>
<td></td>
</tr>
<tr>
<td>Age, years (&gt; 60 vs ≤60)</td>
<td>1.63(0.87-3.03)</td>
<td>0.1256</td>
<td></td>
</tr>
<tr>
<td>Tumor size, cm (&gt; 5 vs ≤5)</td>
<td>1.32(0.67-2.59)</td>
<td>0.4258</td>
<td></td>
</tr>
<tr>
<td>AFP, ng /ml ( &gt;100 vs ≤100)</td>
<td>1.74(0.93-3.26)</td>
<td>0.0814</td>
<td></td>
</tr>
<tr>
<td>HCV genotype, (GT1 vs non-GT1)</td>
<td>1.53(0.85-2.73)</td>
<td>0.1564</td>
<td></td>
</tr>
</tbody>
</table>

HCV, hepatitis C virus; AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma; AST, aspartate aminotransferase; ALT, alanine aminotransferase; SD, standard deviation; Pathological stage according to AJCC and UICC, 7th ed.

*P-value <0.05 is considered significant.

**Table 3.** Univariate and multivariate analysis of factors related to HCC recurrence in HCV-HCC patients.
HR, hazard ratio; CI, confidence interval; HCV, hepatitis C virus; AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Pathological stage according to AJCC and UICC, 7th ed.*P-value <0.05 is considered significant.

Table 4. Univariate and multivariate analysis of factors related to the mortality in HCV-HCC patients.

<table>
<thead>
<tr>
<th>Clinical character Variable</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>Tumor/ANT GHR ratio, (≦0.1 vs &gt; 0.1)</td>
<td>3.60(1.99-6.50)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Gender, (Male vs Female)</td>
<td>0.61(0.34-1.09)</td>
<td>0.0925</td>
</tr>
<tr>
<td>Age, years (&gt; 60 vs ≦60)</td>
<td>2.44(1.27-4.68)</td>
<td>0.0075*</td>
</tr>
<tr>
<td>Tumor size, cm (&gt; 5 vs ≦5)</td>
<td>1.67(0.88-3.16)</td>
<td>0.1168</td>
</tr>
<tr>
<td>Measure</td>
<td>HR (95% CI)</td>
<td>P-value</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>-------------</td>
<td>---------</td>
</tr>
<tr>
<td>AFP, ng/ml (&gt;100 vs ≤100)</td>
<td>1.36(0.76-2.44)</td>
<td>0.2954</td>
</tr>
<tr>
<td>HCV genotype, (GT1 vs non-GT1)</td>
<td>1.35(0.76-2.38)</td>
<td>0.3068</td>
</tr>
<tr>
<td>HCV treatment, (Yes vs No)</td>
<td>0.59(0.43-1.21)</td>
<td>0.2793</td>
</tr>
<tr>
<td>Cirrhosis, (Yes vs No)</td>
<td>1.38(0.78-2.41)</td>
<td>0.2668</td>
</tr>
<tr>
<td>Vascular invasion, (Yes vs No)</td>
<td>2.22(1.26-3.93)</td>
<td>0.0061*</td>
</tr>
<tr>
<td>Pathological Stage (II-IV vs I)</td>
<td>3.34(1.82-6.14)</td>
<td>0.0001*</td>
</tr>
<tr>
<td>AST, IU/L (&gt; 40 vs ≤40)</td>
<td>1.13(0.62-2.05)</td>
<td>0.6979</td>
</tr>
<tr>
<td>ALT, IU/L (&gt; 60 vs ≤60)</td>
<td>1.12(0.64-1.97)</td>
<td>0.6853</td>
</tr>
<tr>
<td>BMI, kg/m² (&gt; 25 vs ≤25)</td>
<td>0.84(0.46-1.54)</td>
<td>0.5739</td>
</tr>
<tr>
<td>Albumin, g/dL (&gt; 4.0 vs ≤ 4.0)</td>
<td>0.34(0.36-1.13)</td>
<td>0.1200</td>
</tr>
</tbody>
</table>

HR, hazard ratio; CI, confidence interval; HCV, hepatitis C virus; AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Pathological stage according to AJCC and UICC, 7th ed.

*P-value <0.05 is considered significant.

Acknowledgements

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Authors contributions
All authors contributed to the study conception and design. C-C Lin, W-L Chuang, C-Y Dai, and M-L Yu conceived and designed the study. C-C Lin, T-W Liu, C-Y Dai, M-L Yu contributed to conducting the experiments. T-W Liu, M-L Yeh, Y-S Tsai, P-C Tsai, C-F Huang, J-F Huang, C-Y Dai, W-L Chuang, M-L Yu contributed to analyzing and interpreting the data. C-C Lin drafted the manuscript. C-Y Dai, and M-L Yu critically revised the article. W-L Chuang, C-Y Dai, and M-L Yu supervised the research. All authors reviewed the results and approved the final version of the manuscript.

Compliance with ethical standards
This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital (ethic approval number: KMUHIRB-20140118) and the TLCN User Committee.

Consent for publication
Not Applicable.

Competing interests
Research support (grant) from Abbott, BMS, Gilead and Merck, Consultant of Abbvie, Abbott, Ascleitis, BMS, Gilead, Merck and Roche. Speaker of Abbvie, Abbott, BMS, Gilead, Merck, and IPSEN. The remaining authors have no conflicts of interest to declare.

References


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**Legends**

**Figure 1.** The expression of GHR in HCV-infected Huh7.5.1 cell model. (A) The efficient production of infectious HCV gradually increased at different time points (2D, 3D and 4D) as detected by immunostaining. Red dash line ranges indicate the HCV-infected cells. Anti-HCV Core protein (green), nuclei (DAPI, blue).
(B) The expression of HCV 5′UTR, NS5B, and GHR detected at different time points (2D, 3D and 4D) in HCV-infected Huh7.5.1 cells by RT-qPCR. The values indicate the mean ± s.d. for three separate experiments (*P < 0.05, **P < 0.01). (C) GHR was highly decreased in HCV-infected cells (red dash line squares) and white line squares indicate non-infected cells. Anti-HCV Core protein (red), anti-GHR (green), nuclei (DAPI, blue). The meaning of 2D, 3D and 4D is Day 2, Day 3 and Day 4, respectively. 5′UTR: 5′ untranslated region, NS5B: nonstructural protein 5B, GHR: growth hormone receptor.

**Figure 2.** The HCV effects on GHR expression and GH/JAK2/STAT5/IGF-1 signaling in the HCV-infected Huh7.5.1 cell model. (A) Immunostaining detected the effect of daclatasvir (DCV) on HCV replication. Twenty-four hours after HCV infection, DCV treatment continued for 3 days. Red dash line ranges indicate the HCV-infected cells. Anti-HCV Core protein (green), nuclei (DAPI, blue). (B) The expression levels of 5′UTR, NS5B, and GHR were detected on Day 4 in HCV-infected Huh7.5.1 cells by RT-qPCR. The values indicate the mean ± s.d. for three separate experiments (**P < 0.01). (C) Western blot analyses for GHR, STAT5, p-STAT5, and HCV Core protein in HCV-infected Huh7.5.1 cells respectively. β-actin was used as a loading control. The data are representative of three independent experiments. The meaning of 3D and 4D is Day 3 and Day 4.

**Figure 3.** The comparison of the recurrent and overall survival (OS) rates after surgery among patients with tumor/ANT GHR ratio $\leq 0.1$ and tumor/ANT GHR ratio $> 0.1$. (A) The recurrent rate of patients with tumor/ANT GHR ratio $\leq 0.1$ was significantly higher than that with the tumor/ANT GHR ratio $> 0.1$ ($p < 0.0001$). (B) OS of patients with tumor/ANT GHR ratio $\leq 0.1$ was significantly shorter than that with the tumor/ANT GHR ratio $> 0.1$ ($p < 0.0001$). (C, D) Tumor/ANT GHR ratio $\leq 0.1$ in HCV-HCC patients with age older than 60 years showed apparent prognostic value in predicting the higher recurrent rate and poorer OS. (E, F) Tumor/ANT GHR ratio $\leq 0.1$ in HCV-HCC patients with pathological stages II-IV was significantly correlated with higher recurrent rate and shorter OS. The solid line represented patients
with a tumor/ANT GHR ratio of $\leq 0.1$. The dotted line represented patients with tumor/ANT GHR ratio $> 0.1$.

**Table 1.** Clinicopathological information of HCV-related HCC patients obtained from the TLCN

**Table 2.** Clinical characteristics of HCV-HCC patients according to the ratio of tumor/ANT GHR expression (tumor/non-tumor part)

**Table 3.** Univariate and multivariate analysis of factors related to HCC recurrence in HCV-HCC patients.

**Table 4.** Univariate and multivariate analysis of factors related to the mortality in HCV-HCC patients.