OncoTargets and Therapy

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

Overexpression of *GIHCG* is Associated with a Poor Prognosis and Immune Infiltration in Hepatocellular Carcinoma

This article was published in the following Dove Press journal:
OncoTargets and Therapy

Siyu Xiao[1,*]
Shanzhou Huang[2,*]
Jie Yang[3]

[1]Department of Clinical Laboratory, The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou, People's Republic of China; [2]Department of General Surgery, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, School of Medicine, South China University of Technology, Guangzhou, People's Republic of China; [3]Guangdong Lung Cancer Institute, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, School of Medicine, South China University of Technology, Guangzhou, People's Republic of China

*These authors contributed equally to this work

Purpose: Hepatocellular carcinoma (HCC) is one of the most commonly diagnosed digestive cancers and the fourth leading cause of death worldwide. Long noncoding RNAs (lncRNAs) with key roles in HCC development and progression have emerged and are used in the diagnosis and prognostic prediction of HCC. The lncRNA gradually increased during hepatocarcinogenesis (*GIHCG*) is a novel lncRNA with aberrant expression in many tumors, but its prognostic value and biological role in HCC have not been fully studied. Thus, the aim of this study was to explore the expression pattern and potential biological role of *GIHCG* in HCC.

Patients and Methods: The expression pattern of *GIHCG* in HCC was analyzed in our HCC cohort and validated in The Cancer Genome Atlas (TCGA) database. To assess the prognostic value of *GIHCG*, survival analyses and Cox regression analyses were carried out in two HCC cohorts. Functional enrichment analysis was used to predict the gene sets and pathways related to aberrant *GIHCG* expression. Furthermore, the relationship between *GIHCG* expression and immune infiltration in HCC was analyzed.

Results: *GIHCG* was highly expressed in HCC tissues compared with normal liver tissues. In addition, high *GIHCG* expression was significantly correlated with inferior clinicopathological characteristics and shorter survival times. High *GIHCG* expression was an independent prognostic factor for overall survival and disease-free survival in the HCC cohort in our center and in the TCGA-LIHC cohort. Hallmark terms such as "G2M checkpoint", "*MYC* targets" and "DNA repair" were enriched in the *GIHCG* high-expression groups. High *GIHCG* expression negatively correlated with the infiltration of memory CD4+ and CD8+ T cells, natural killer cells, macrophages, dendritic cells, neutrophils and monocytes.

Conclusion: The findings of our study indicate that *GIHCG* is a biomarker that can be used to predict the prognosis of HCC patients and is correlated with immune cell infiltration in HCC.

Keywords: *GIHCG*, prognosis, immune cell infiltration

Introduction

Hepatocellular carcinoma (HCC) is the third most common digestive cancer and the fourth leading cause of cancer death worldwide.[1] The 5-year survival rate of HCC is lower than 20%,[2] which is primarily attributed to the difficulty of early diagnosis via current biomarkers.[3] As a result, most HCC patients are diagnosed at an advanced stage, at which point the opportunity to receive curative treatments is lost.[4,5] Moreover, even for those undergoing surgical resection, recurrence remains a major problem, and half of the patients with recurrence die within 1 year. Thus, an urgent need to develop

Correspondence: Jie Yang
Guangdong Lung Cancer Institute, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, School of Medicine, South China University of Technology, No. 106, 2nd Zhongshan Road, Guangzhou, People's Republic of China
Email yj12666666@126.com

submit your manuscript | www.dovepress.com
DovePress
http://doi.org/10.2147/OTT.S271966

new approaches for early diagnosis, real-time monitoring, and effective treatment of HCC remains.

Long noncoding RNAs (lncRNAs) make up a large class of noncoding transcripts that are usually greater than 200 nucleotides in length. Multiple studies have reported that aberrant lncRNA expression affects tumor cell growth, apoptosis, invasion, and metastasis. LncRNAs may be useful in cancer diagnosis and prognostic evaluations and some functional lncRNAs may serve as potential therapeutic targets based on their key roles in vital biological processes of tumor development and progression.[6–12]

One lncRNA known as gradually increased during hepatocarcinogenesis (*GIHCG*) is a functional lncRNA that was recently identified by Sui et al in HCC.[13] The *GIHCG* gene alias is *GIHCG* inhibitor of miR-200b/200a/429 expression. The *GIHCG* gene has 4727 bases and is located on 12q14.1, and the Ensembl ID of *GIHCG* is ENSG00000257698 (Ensembl version: ENSG00000257698.2). The former transcript name was RP11-620J15.3 (Ensembl version: ENSG00000257698.1). By epigenetically inhibiting miR-200b/a/429 expression, Sui et al showed that *GIHCG* affects the proliferation, migration, and invasion of HCC cell lines.[13] In addition, *GIHCG* has been reported to be overexpressed in and promote tumor progression of renal cell carcinoma, tongue squamous cell carcinoma, ovarian cancer, cervical cancer, breast cancer, colorectal cancer and gastric cancer.[14–21]

We hypothesized that *GIHCG* may act as an oncogene during tumorigenesis, however the prognostic value of *GIHCG* and its possible biological functions in HCC have yet to be fully assessed. Our study aims to assess the prognostic value of *GIHCG* in both the public HCC database and our clinical HCC cohort and to explore potential pathway correlations with *GIHCG* expression.

Patients and Methods

SYSU HCC Sample Collection and Follow-Up

The present study included 120 patients with HCC who underwent R0 surgical resection between July 2013 and December 2014 from the First Affiliated Hospital, Sun Yat-sen University (SYSU). One hundred HCC samples were formalin-fixed and paraffin-embedded as the main evaluation cohort. The other 20 fresh HCC tissues with paired adjacent normal tissue (ANT) samples were obtained and stored in RNAlater solution (Invitrogen, USA) immediately after resection and then frozen in liquid nitrogen until further use, as previously described.[22] None of the enrolled patients received any radiotherapy or chemotherapy before surgery, and all specimens were confirmed by pathology. Data on the clinical and pathological characteristics, such as tumor stage, differentiation grade, presentation of tumor thrombi, HBsAg status and AFP level, were retrieved from the inpatient database. Overall survival (OS) was defined as the period from resection to death or the last follow-up. Disease-free survival (DFS) was defined as the period between surgery and death or first confirmed relapse until the last follow-up date in December 2018.

This study conformed to the 1964 Declaration of Helsinki and was approved by the Ethics Committee of the First Affiliated Hospital, Sun Yat-sen University. Written informed consent was obtained from all patients for the use of their tissue samples.

RNA Extraction and *GIHCG* Expression Measurement

Total RNA was extracted from approximately 100 mg of tissue using the Qiagen RNeasy Mini Kit according to the manufacturer's instructions as previously described.[23] To detect *GIHCG* expression in HCC and ANTs, Quantitative real-time PCR (qRT-PCR) was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, USA) according to the manufacturer's instructions with gene-specific primers. Primers were designed as follows:

GIHCG forward primer, 5′-CTTTCAAGAAGTTTG GCTGTC-3′; *GIHCG* reverse primer: 5′-GCTCATTCAA CGGATAAGTC-3′; *GAPDH* forward primer: 5′-TGTGG GCATCAATGGATTTGG-3′; *GAPDH* reverse primer: 5′-ACACCATGTATTCCGGGTCAAT-3′.

Chromogenic in situ Hybridization (CISH)

To evaluate the expression level of *GIHCG*, CISH was applied to SYSU HCC samples. All slices were fixed for 2–12 hrs with 4% paraformaldehyde (DEPC, Servicebio, Wuhan, China), incubated in proteinase K (Servicebio) for 30 mins and rinsed three times with PBS (Servicebio). Next, the samples were prehybridized in hybridization buffer (Servicebio) at 37°C for 1 hr. Then, fresh hybridization buffer containing 8 ng/mL of the corresponding probe was used to replace the prehybridization buffer, and the samples were incubated at 37°C overnight. Finally, nuclear fast red

(Servicebio) staining was performed to visualize the cell nuclei of the samples, and the samples were mounted in neutral balsam (Sinopharm Chemical Reagent Co., Ltd) and examined by bright-field microscopy. Based on the staining intensity, the patients were divided into high- or low-expression groups as previously described.[22]

Public Datasets Download and Validation

To determine the expression profile of *GIHCG* in HCC, we downloaded the expression data, clinical information and mutation data from The Cancer Genome Atlas (TCGA) Liver Hepatocellular Carcinoma (TCGA-LIHC) dataset (April 21, 2020) using the R package *TCGAbiolinks*[24] (version 2.14.0). *GIHCG* expression levels were compared using transcripts per kilobase million (TPM) normalization. After exclusion of repeat samples from the same patient, 371 HCC and 50 paired normal liver tissues were included for expression analysis. In addition, patients with any missing or insufficient data on TNM stage, OS or DFS were excluded from the survival analysis and correlation analysis of *GIHCG* with clinicopathologic characteristics. Finally, a total of 347 HCC patients were included for the correlation and survival analysis. High- and low-expression patients were divided according to the median expression level of *GIHCG*. Because a limited number of normal liver samples were available in the TCGA-LIHC dataset, the expression of *GIHCG* in HCC and normal liver tissue was further validated in GEPIA, a web server for analyzing the RNA-sequencing data from TCGA and thousands of normal samples from GTEx projects (http://gepia.cancer-pku.cn).

Functional Enrichment Analysis for *GIHCG*

Differentially expressed genes between the high- and low-expression groups were identified using the R package Limma[25] (version 3.42.2). Gene Set Enrichment Analysis (GSEA) and Gene Ontology (GO) analyses were conducted

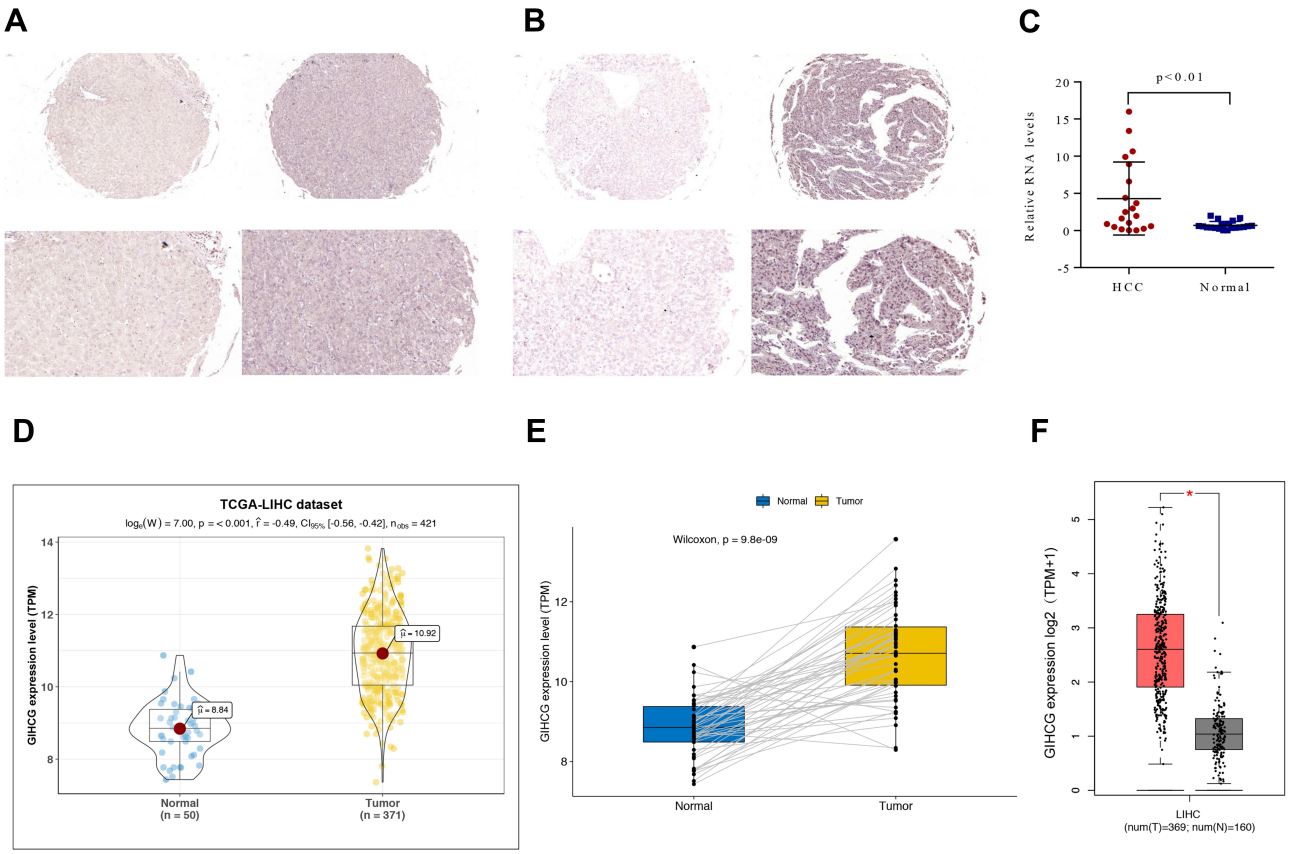

Figure 1 *GIHCG* expression in HCC and normal liver samples. (**A** and **B**) Representative photos (magnification: 20× and 40×) of *GIHCG* expression in adjacent normal liver tissues (**A**) and HCC (**B**) tissues. (**C**) *GIHCG* expression was examined by qRT-PCR in 20 paired HCC tissues and adjacent normal liver tissues. (**D** and **E**) Comparison of *GIHCG* expression in HCC and normal liver using the TCGA-LIHC cohort containing 371 HCC samples and 50 paired adjacent normal liver tissue samples as shown in Panel B; (**F**) Comparison expression of *GIHCG* in HCC (TCGA-LIHC: 369 HCC samples, excluding 2 repeated patients) and normal liver (GETx: 160 donation liver samples).

with the R package (clusterProfiler,[26] version 3.14.3) to explore different molecular mechanisms and involved pathways between high- and low-expression groups in the TCGA-LIHC dataset. Hallmark gene sets and molecular signatures were obtained from the Molecular Signatures Database. Normalized enrichment scores were acquired using gene set permutations 1000 times, and a cutoff P-value of 0.05 was used to filter the significant enrichment results.

Immune Cell Infiltrates Analysis

To evaluate the correlation between *GIHCG* expression and tumor-infiltrating immune cells (TIICs), we performed single-sample gene set enrichment analysis (ssGSEA),[27] a method used to transform the bulk transcriptomic expression data into relative abundance of specific immune cell types, to quantitatively identify the proportion of TIICs in the tumor microenvironment. Feature gene panels for each immune cell type were obtained and referenced from recent publications.[28,29] The ssGSEA enrichment scores of each immune cell type were calculated by using TCGA-LIHC expression data and R package GSVA[30] (version 1.34.0). The immune cell infiltration profiles and marker genes of different immune cell subsets was compared between the *GIHCG* high- and low-expression groups. For heatmap visualization, the ssGSEA scores were normalized to show the relative abundance of infiltrating immune cell populations.

Statistical Analysis

All statistical analyses were performed using R software (version 3.6.2) with the corresponding packages. The Shapiro–Wilk method was used for normality tests. The mean ± standard deviation (mean ± SD) is presented for normally distributed variables, and the median is presented for nonnormally distributed variables. The relationships between categorical variables were compared using the $\chi 2$ test, and continuous variables were analyzed with Student's *t*-test. Kaplan-Meier survival analysis and the Log-rank test were employed to analyze OS and DFS (R package survival, version 3.1–8). Univariate and multivariate Cox regression analyses were performed to evaluate the prognostic value of *GIHCG*.

Results

GIHCG Expression in HCC and Normal Tissues

GIHCG expression was first investigated in the SYSU HCC cohort. CISH staining revealed that HCC tissues exhibited significantly higher *GIHCG* expression than the adjacent normal liver tissue. Representative photos showed *GIHCG* low- and high-expression in normal liver tissue (Figure 1A) and HCC (Figure 1B). The relative *GIHCG* mRNA expression was significantly higher in HCC tissues compared to normal tissues (Figure 1C).

This expression pattern was then validated in 371 HCC and 50 adjacent normal liver tissues using the TCGA-LIHC

Table 1 Association Between *GIHCG* Expression and the Clinicopathological Characteristics of Patients in SYSU HCC Cohort

Variables	Total (n)	Low Expression (n=42)	High Expression (n=58)	*P* value
Gender				0.126
Male	86	33 (78.6)	53 (91.4)	
Female	14	9 (21.4)	5 (8.6)	
Age, years				0.126
<50	49	26 (61.9)	23 (39.7)	
≥50	51	16 (38.1)	35 (60.3)	
Tumor nodule number				0.006
Solitary	59	32 (76.2)	27 (46.6)	
Multiple (≥2)	41	10 (23.8)	31 (53.4)	
Tumor size (cm)				<0.001
≤5	50	31 (73.8)	19 (32.8)	
>5	50	11 (26.2)	39 (67.2)	
Cancer embolus				<0.001
Absence	66	35 (83.3)	31 (53.4)	
Presence	34	7 (16.7)	27 (46.6)	
AFP, ng/mL				<0.001
<200	52	31 (73.8)	21 (36.2)	
≥200	48	11 (26.2)	37 (63.8)	
HBsAg				0.724
Negative	42	19 (45.2)	23 (39.7)	
Positive	58	23 (54.8)	35 (60.3)	
TNM stage				0.098
Stage I & II	51		26 (61.9)	25 (43.1)
Stage III & IV	49	16 (38.1)	33 (56.9)	
Differentiation grade				0.234
Well	66	31 (73.8)	35 (60.3)	
Poor	34	11 (26.2)	23 (39.7)	

dataset. In the TCGA-LIHC cohort, *GIHCG* expression was significantly higher in tumors than in normal tissues (P-value < 0.001). *GIHCG* expression in HCC and normal liver samples is plotted in Figure 1D. Furthermore, in the 50 paired adjacent normal tissues from the same HCC patients, *GIHCG* expression was significantly upregulated in the HCC tissues compared with the adjacent normal liver tissues (Figure 1E). Using the online GEPIA databases, which included many more normal liver tissues from the GTEx projects, *GIHCG* expression was also significantly upregulated in HCC tissues (n = 369) compared with normal liver tissues (n = 160, Figure 1F). These results demonstrate that *GIHCG* is highly expressed in HCC tissues compared to normal tissues.

Correlation of *GIHCG* Expression with Clinicopathological Characteristics

To investigate the association between *GIHCG* expression and clinical factors in HCC patients, we analyzed the *GIHCG* expression in different clinicopathological subgroups in both the SYSU HCC and TGCA-LIHC cohorts. We performed CISH staining via semiquantitative measurement of *GIHCG* in the SYSU HCC cohort. Within all the SYSU HCC samples, 58 (58.0%) displayed high *GIHCG* expression. The relationship between the *GIHCG* expression level and HCC clinicopathological features is shown in Table 1. The results showed that *GIHCG* expression was significantly associated with tumor numbers (P < 0.05), tumor size (P < 0.05), AFP level and tumor thrombus (P < 0.05).

In the TCGA-LIHC cohort, as shown in Figure 2, *GIHCG* overexpression was positively associated with the TNM stage, histological grade and T stage (Figure 2A–C); however, other clinicopathologic factors, such as gender, AFP levels and status of hepatitis virus infection, had no significant association with *GIHCG* expression (data not shown). In addition, *GIHCG* high expression was significantly correlated with TP53 mutation (Figure 2D).

High *GIHCG* Expression Predicts Poor Survival Among Patients with HCC

To verify the prognostic value of *GIHCG* expression in HCC, we performed a Kaplan-Meier analysis in the SYSU HCC cohort and TCGA-LIHC cohort. In the SYSU HCC

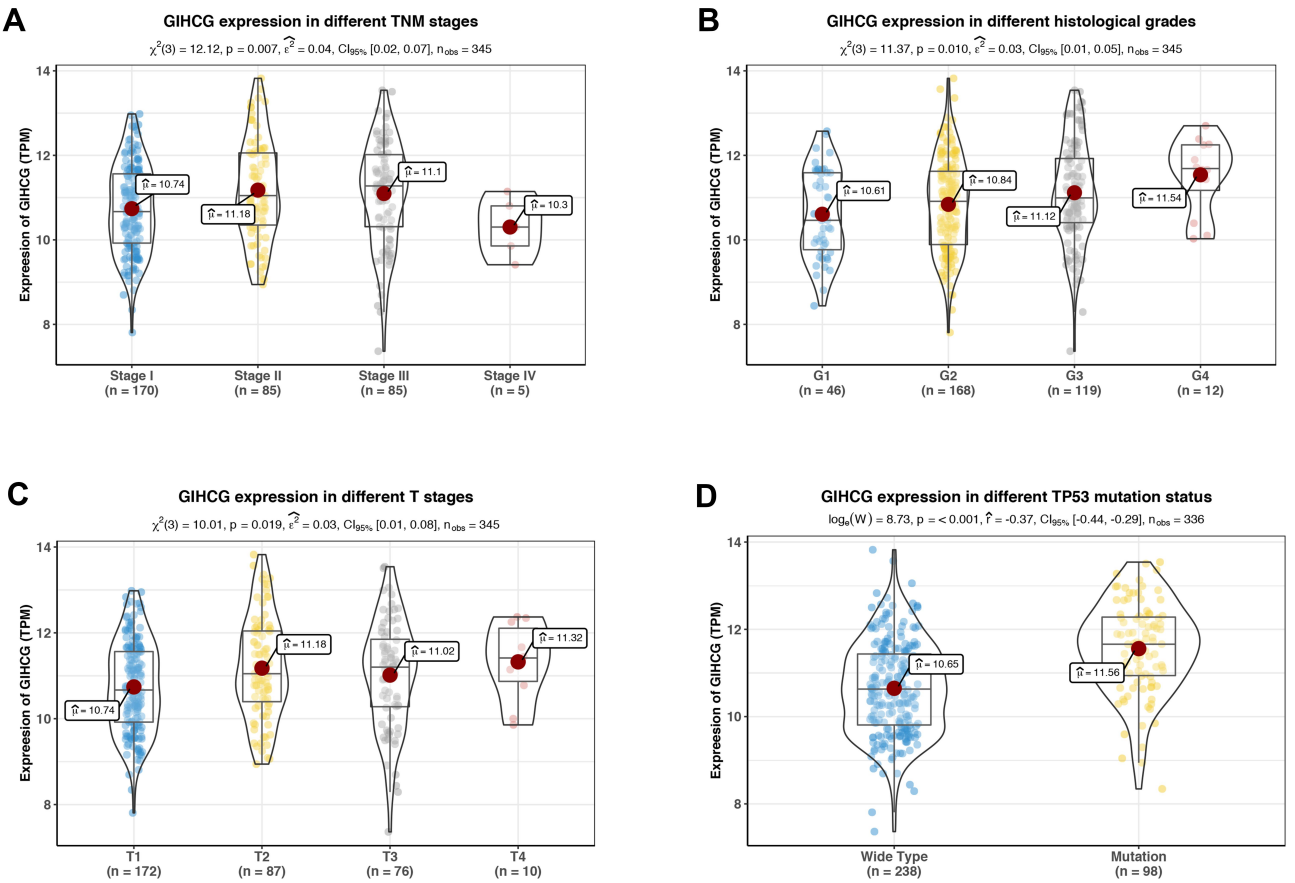

Figure 2 Correlation of *GIHCG* expression with clinical and pathological characteristics. *GIHCG* expression profiles in different TNM stages (**A**), histological grades (**B**), T stages (**C**) and TP53 mutation status (**D**). *GIHCG* expression is significantly correlated to tumor stage, tumor grade, tumor size and TP53 mutation in HCC.

cohort, the median OS time of the low-*GIHCG*-expression group was significantly longer than that of the high-*GIHCG*-expression group (HR=0.269, 95% CI: 0.133–0.544; P<0.001; Figure 3A), and the median DFS of the low-*GIHCG*-expression group was significantly longer than that of the high-*GIHCG*-expression group (HR=0.262, 95% CI: 0.129–0.530; P<0.001; Figure 3B). Consistent with the SYSU HCC cohort, in the TCGA-LIHC cohort, the median OS time of the low-*GIHCG*-expression group was significantly longer than that of the high-*GIHCG*-expression group (HR=0.537, 95% CI: 0.-378–0.763; P<0.001; Figure 3C), and the median DFS time of the low-*GIHCG*-expression group was significantly longer than that of high-*GIHCG*-expression group (HR=0.614, 95% CI: 0.457–0.825; P<0.001; Figure 3D).

Moreover, univariate and multivariate Cox regression analysis was performed to determine the relationships between risk factors and prognosis in the two HCC cohorts. The results revealed that tumor nodule number, tumor size, TNM stage, histological grade, tumor thrombi, AFP level and *GIHCG* expression level were significantly associated with OS and DFS in the SYSU cohort. In the TCGA-LIHC cohort, TNM stage, T stage, hepatitis virus infection and *GIHCG* expression were significantly associated with OS and DFS (Tables 2 and 3).

These significant prognostic risk factors were included in the multivariate Cox regression analysis. The results showed that in the SYSU cohort, tumor thrombi (HR=2.044; 95% CI: 1.023–4.085; P=0.043), TNM stage (HR=2.245; 95% CI: 1.-14–4.421; P=0.019) and *GIHCG* expression level (HR=0.333; 95% CI: 0.146–0.756; P= 0.009) were independent prognostic factors for OS (Table 2, Supplementary Figure 1A). Further, TNM stage (HR=2.465; 95% CI: 1.233–4.926; P=0.011) and *GIHCG* expression level (HR=0.365; 95% CI: 0.161–0.828; P= 0.016) were independent prognostic factors for DFS (Table 3, Supplementary Figure 1B). In the TCGA-LIHC cohort, hepatitis virus infection (HR=1.979; 95% CI: 1.356–-2.887; P< 0.001) and *GIHCG* expression (HR=0.551; 95% CI:

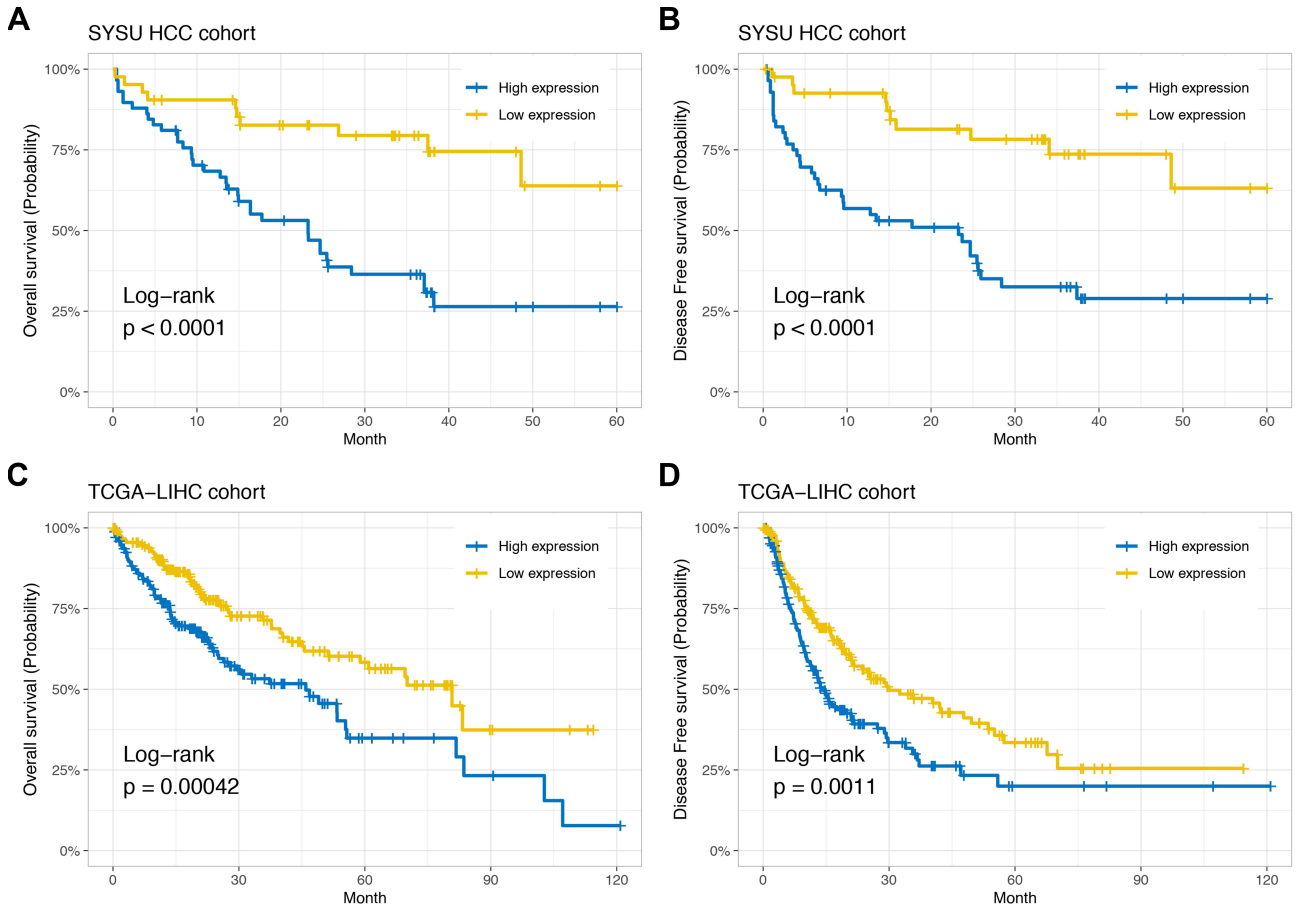

Figure 3 High *GIHCG* expression is associated with poor prognosis. Kaplan-Meier curves show overall survival (**A**) and disease-free survival (**B**) curves for 58 *GIHCG* high-expression patients and 42 *GIHCG* low-expression patients in the SYSU HCC cohort. Kaplan-Meier plots show overall survival (**C**) and disease-free survival (**D**) curves for 185 *GIHCG* high-expression patients and 186 *GIHCG* high-expression patients from the TCGA-LIHC cohort.

Table 2 Univariate and Multivariate Cox Regression Analyses of Risk Factors Associated with Overall Survival in SYSU HCC and TCGA-LIHC Cohort

Variables	Univariate Analysis		Multivariate Analysis	
SYSU HCC cohort (n=100)	HR (95% CI for HR)	P value	HR (95% CI for HR)	P value
Gender	3.19 (0.985–10.3)	0.053		
Age	1.21 (0.679–2.15)	0.520		
TNM stage	2.95 (1.61–5.41)	0.000	2.245 (1.14–4.421)	0.019
HBV infection	1.55 (0.846–2.83)	0.157		
Tumor number	0.498 (0.28–0.884)	0.017	1.182 (0.591–2.364)	0.636
Tumor size	2.65 (1.42–4.95)	0.002	1.381 (0.708–2.694)	0.344
Tumor thrombus	3.27 (1.84–5.82)	< 0.001	2.044 (1.023–4.085)	0.043
AFP level	2.11 (1.17–3.79)	0.013	0.931 (0.462–1.874)	0.841
Differentiation grade	0.506 (0.285–0.898)	0.020	0.784 (0.426–1.443)	0.435
GIHCG expression level	0.269 (0.133–0.544)	< 0.001	0.333 (0.146–0.756)	0.009
TCGA LIHC cohort (n=345)	HR (95% CI for HR)	P value	HR (95% CI for HR)	P value
Gender	0.776 (0.531–1.13)	0.188		
Age	1.19 (0.748–1.9)	0.459		
TNM stage	2.5 (1.72–3.63)	< 0.001	0.769 (0.105–5.656)	0.797
T stage	2.52 (1.73–3.67)	< 0.001	2.893 (0.393–21.299)	0.297
HBV/HCV infection	2 (1.38–2.91)	< 0.001	1.979 (1.356–2.887)	< 0.001
AFP level	0.984 (0.597–1.62)	0.949		
Differentiation grade	1.14 (0.784–1.66)	0.490		
GIHCG expression	0.518 (0.356–0.754)	< 0.001	0.551 (0.376–0.807)	0.002

Table 3 Univariate and Multivariate Cox Regression Analyses of Risk Factors Associated with Disease-Free Survival in SYSU HCC and TCGA-LIHC Cohort

Variables	Univariate Analysis		Multivariate Analysis	
SYSU HCC cohort (n=100)	HR (95% CI for HR)	P value	HR (95% CI for HR)	P value
Gender	2.28 (0.816–6.39)	0.116		
Age	1.11 (0.621–1.98)	0.728		
TNM stage	3.26 (1.76–6.06)	< 0.001	2.465 (1.233–4.926)	0.011
HBV infection	1.54 (0.838–2.82)	0.164		
Tumor number	0.396 (0.221–0.711)	0.002	0.759 (0.385–1.498)	0.427
Tumor size	3.41 (1.77–6.59)	< 0.001	1.924 (0.966–3.835)	0.063
Tumor thrombus	2.96 (1.65–5.3)	< 0.001	1.379 (0.704–2.701)	0.349
AFP level	2.36 (1.3–4.3)	0.005	0.915 (0.44–1.904)	0.813
Differentiation grade	0.488 (0.273–0.87)	0.015	0.86 (0.447–1.653)	0.65
GIHCG expression level	0.262 (0.129–0.53)	< 0.001	0.365 (0.161–0.828)	**0.016**
TCGA LIHC cohort (n=345)	HR (95% CI for HR)	P value	HR (95% CI for HR)	P value
Gender	0.933 (0.673–1.29)	0.679		
Age	0.991 (0.682–1.44)	0.963		
TNM stage	2.21 (1.6–3.07)	< 0.001	1.312 (0.318–5.415)	0.708
T stage	2.21 (1.59–3.07)	< 0.001	1.573 (0.378–6.542)	0.534
HBV/HCV infection	1.45 (1.07–1.98)	0.018	1.379 (1.009–1.884)	0.044
AFP level	0.971 (0.651–1.45)	0.886		
Differentiation grade	1.15 (0.841–1.57)	0.380		
GIHCG expression	0.619 (0.454–0.844)	0.002	0.666 (0.486–0.911)	0.011

0.376–0.807; P=0.002) were independent prognostic factors for OS (Table 2, Supplementary Figure 1C) and hepatitis virus infection (HR=1.379; 95% CI: 1.009–1.884; P=0.044) and *GIHCG* expression (HR=0.666; 95% CI: 0.486–0.911; P=0.011) were independent prognostic factors for DFS (Table 3, Supplementary Figure 1D).

To test the robustness of *GIHCG* expression for the prediction of HCC survival, we validated the prognostic significance in different clinicopathological subgroups. The result of the subgroups analysis showed that *GIHCG* expression can also serve as a prognostic factor for OS and DFS in most subgroups (Figure 4, Supplementary Figure 2–5). In summary, the above results show that high *GIHCG* expression is positively correlated with poor survival among HCC patients.

Functional Enrichment Analysis of High *GIHCG* Expression

To evaluate potential key molecules and pathways associated with *GIHCG* expression, we performed GO analysis and GSEA between high- and low-expression groups. The top enriched GO terms in biological processes (BP), cellular components (CC) and molecular function (MF) are shown in Figure 5A and Supplementary Table 1. GO analysis revealed that "RNA catabolic process", "organic acid catabolic process" and "spindle organization" were the main terms involved in BP; "spindle", "chromosomal region" and "cytosolic ribosome" were significantly enriched in CC; and "coenzyme binding", "structural constituent of ribosome" and "catalytic activity, acting on DNA" were the top enriched terms in MF. GSEA enrichment analysis showed that the significantly enriched hallmark terms included "G2M checkpoint", "E2F targets", "spermatogenesis", "*MYC* targets" and "DNA repair" (Figure 5B and C, Supplementary Table 2), which were associated with the proliferation of cancer cells. The results of the KEGG pathway analysis showed that 77 pathways were enriched ($p<0.05$), among which "Cell cycle", "Ribosome", "Fanconi anemia pathway", "Spliceosome" and "DNA

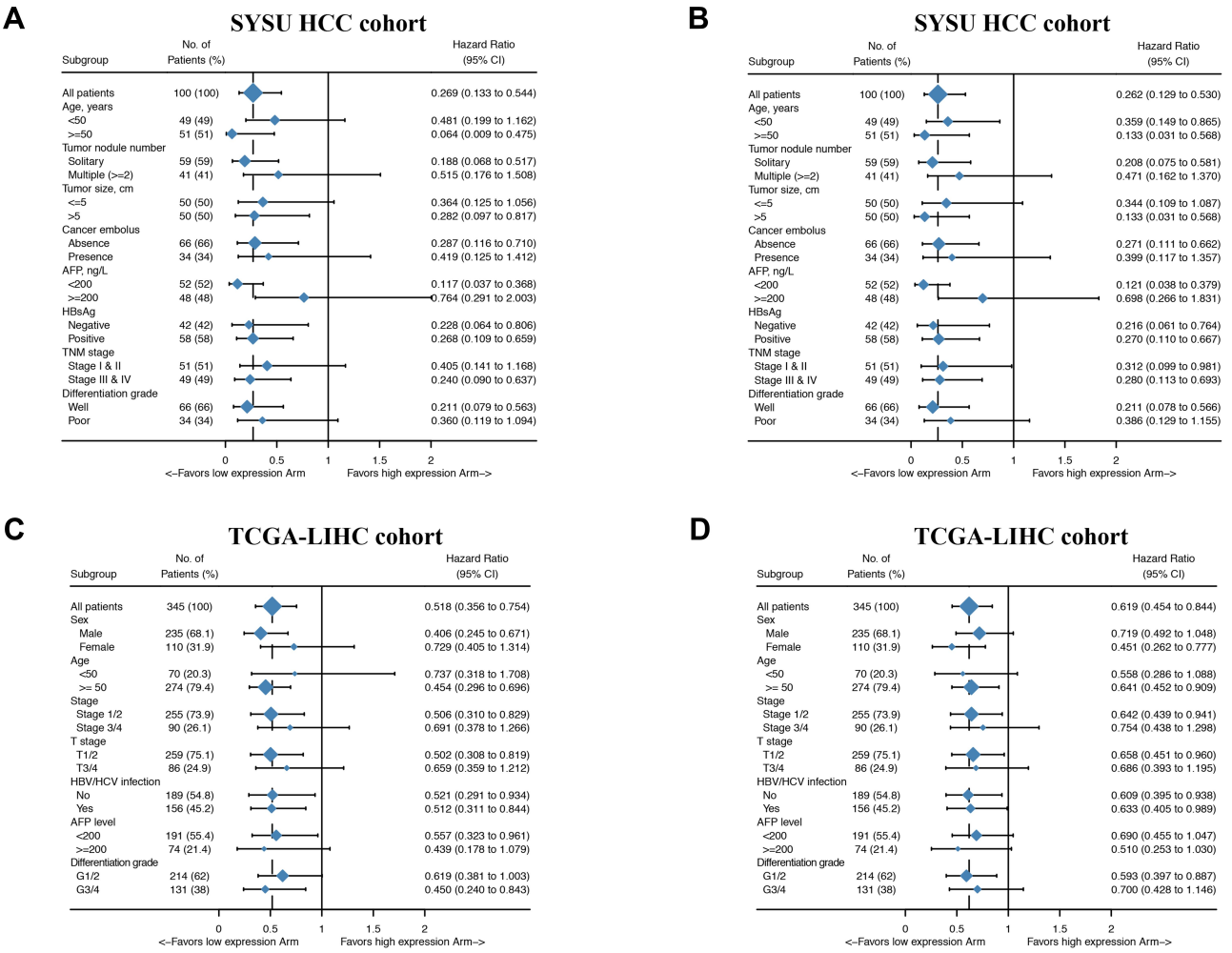

Figure 4 Hazard ratio values for subgroup analyses of *GIHCG* expression in HCC patients. Forest plots summarizing the hazard ratio (HR) between *GIHCG* expression and survival in HCC patients as analyzed by the different clinicopathological subgroups. (**A**) HR values for OS in the SYSU HCC cohort. (**B**) HR values for DFS in the SYSU HCC cohort. (**C**) HR values for OS in the TCGA-LIHC cohort. (**D**) HR values for DFS in the TCGA-LIHC cohort.

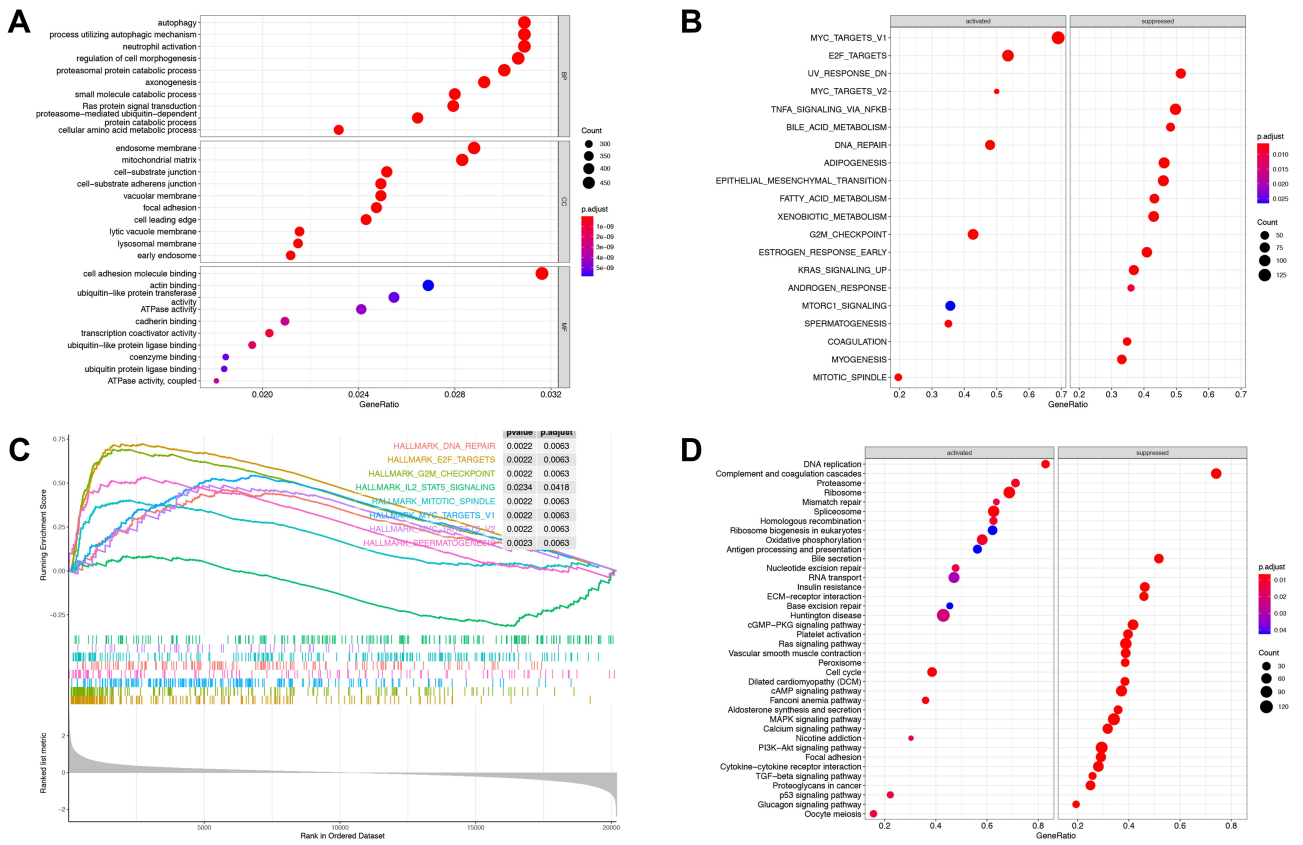

Figure 5 Gene set enrichment analysis between *GIHCG* high- and low-expression groups. (**A**) Gene Ontology enrichment analysis includes biological processes, cellular components and molecular function. (**B**) Enriched hallmark terms correlated to *GIHCG* expression. (**C**) Representative hallmark terms enrichment plot. (**D**) GSEA-based Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed the enriched activated and suppressed pathways between the *GIHCG* high- and low- expression groups. The circle size indicates the counts of enriched terms and the color represents the P value.

replication" were highly associated with high *GIHCG* expression (Figure 5D, Supplementary Table 3).

Correlations Between *GIHCG* Expression and Immune Cell Infiltration and Markers

Because tumor microenvironmental alterations correlate with a poor prognosis among HCC patients,[31–34] we investigated the correlation between the *GIHCG* expression level and the proportions of TIICs in the TCGA-LIHC cohort. Figure 6A shows the relative abundance of 28 TICs populations for 345 HCC tumor samples in the TCGA-LIHC cohort. The results of the ssGSEA showed that antitumor immune cells, including central memory T cells, effector memory T cells, type 1 T helper cells, type 17 T helper cells, $CD56^{bright}$ natural killer cells and natural killer cells, were significantly lower in the high-*GIHCG*-expression group than the low-*GIHCG*-expression group (Figure 6B and Supplementary Table 4). Other TIICs, such as activated CD4 regulatory T cells, $CD56^{dim}$ natural killer cells, neutrophils, plasmacytoid dendritic cells, eosinophils and monocytes, also significantly differed between the high-*GIHCG*- and low-*GIHCG*-expression groups. Specifically, *GIHCG* expression had a significant negative correlation with the proportion of TIICs, including central memory CD4/8 T cells, effector memory CD4/8 T cells, type 1/17 T helper cells, immature dendritic cells, plasmacytoid dendritic cells, neutrophils, eosinophils and monocytes (Figure 7A and Supplementary Table 5). We next explored the correlation between *GIHCG* expression and the marker of genes of different immune cell subsets (Figure 7B and Supplementary Table 6). The *GIHCG* expression level was positively correlated with the gene markers of T cells (*CD8A*, *CD8B*, *CD2*, *CD3D*, *CD3E*), T cell exhaustion (*LAG3*, *CTLA4*, *GZMB*), B cells (*CD19*), macrophages (*CD68*, *IRF5*, *VSIG4*, *MS4A4A*), dendritic cells (*HLA-DPB1*, *HLA-DQB1*, *HLA-DRA*, *ITGAX*), natural killer cells (*KIR2DL4*, *NCAM1*) and neutrophils (*ITGAM*). These results suggest that *GIHCG* expression correlates with the tumor microenvironment in HCC.

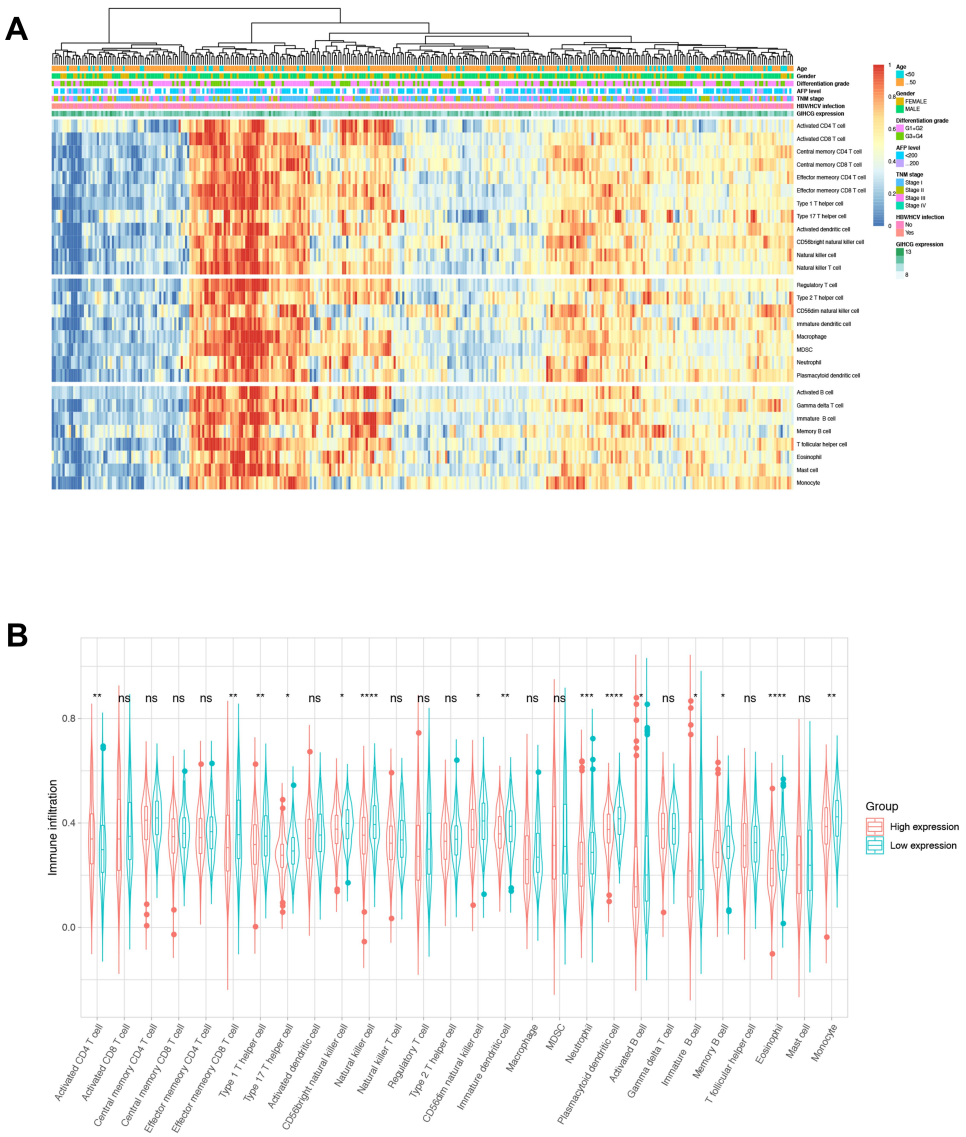

Figure 6 Visualization of the relationships between infiltrating immune cells and *GIHCG* expression. (**A**) A heatmap shows the relative infiltration of immune cell proportion for all TCGA-LIHC tumor patients (normalized ssGESA). *GIHCG* value, age, gender, tumor stage, and AFP are shown as patient annotations. (**B**) Comparison of infiltration immune cells differences in the *GIHCG* high- and low-expression groups. Symbol "****", "***", "**", "*", "ns" indicate a P value <0.0001, <0.001, <0.01, <0.05 and >0.05, respectively.

Discussion

Although many advances have been achieved in the management and treatment of HCC, HCC ranks as the third leading cause of tumor-related death worldwide. Identification of meaningful biomarkers associated with HCC progression are still needed. *GIHCG* acts as an onco-long noncoding RNA in many types of cancer, but its prognostic value, biological functions, and relationship with the tumor microenvironment have not been fully assessed. In this study, we analyzed the prognostic value and potential biological functions of *GIHCG* in HCC.

In the current study, we systematically analyzed the *GIHCG* expression pattern in HCC, and our results demonstrated that *GIHCG* expression is significantly increased in HCC tissues compared with normal liver tissues. High *GIHCG* expression was positively correlated with poor clinicopathologic factors and poor survival among HCC patients. *GIHCG* expression was an independent prognostic factor of OS and DFS for HCC patients. These results were validated in both public databases and local HCC cohorts, and the results were consistent with those of a previous study.[13]

Additionally, bioinformatics results revealed that high *GIHCG* levels activated proliferation pathways in HCC,

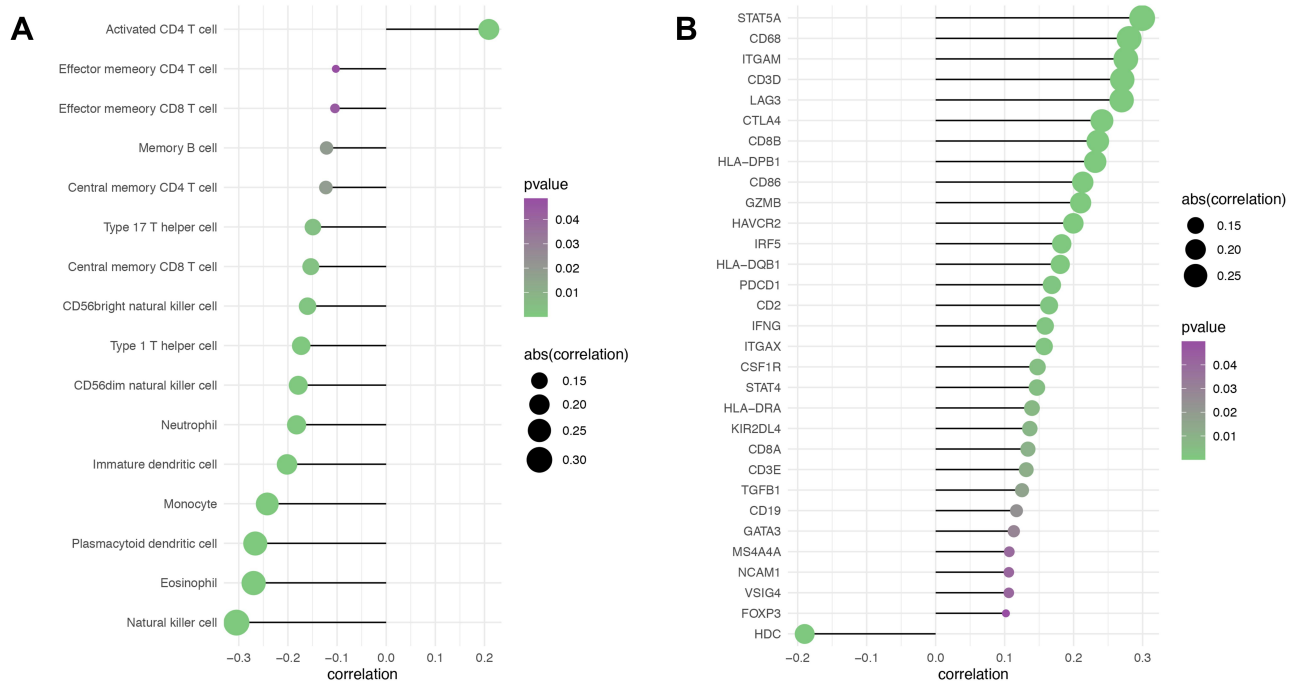

Figure 7 Correlation of *GIHCG* expression and immune cell infiltration in HCC. (**A**) Spearman correlation analysis between *GIHCG* expression and the proportion of immune cell infiltration. (**B**) Correlation analysis between *GIHCG* expression and immune cell marker genes. Only significant results were plotted. The circle size indicates the absolute value of R and the color represents the P value.

which is consistent with the findings of Sui et al showing that *GIHCG* promotes HCC cell proliferation and metastasis in vivo by silencing miR-200b/a/429.[13] The GO terms suggested that *GIHCG* overexpression could alter the RNA catabolic process in HCC. KEGG pathway analysis revealed that "Cell cycle" and "DNA replication" were linked to *GIHCG* overexpression. Previous studies have supported that lncRNAs are involved in vital biological processes, such as the cell cycle and DNA repair.[35,36] GSEA showed that *GIHCG* overexpression is involved in pivotal hallmarks of proliferation, including "G2M checkpoint", "E2F targets", "MYC targets" and "DNA repair". LncRNAs are reported to be regulated by MYC and affect MYC expression in different cancer types, which could influence cancer cell viability and proliferation.[37,38] *GIHCG* may also be involved in these key pathways, but further studies are needed to confirm this hypothesis.

LncRNAs are directly and indirectly involved in the crosstalk between immune cells and tumor cells, and dysregulated lncRNA expression in these cells could drive tumorigenesis in HCC.[39–42] Our results demonstrate that *GIHCG* expression is significantly correlated with the tumor microenvironment in HCC. High *GIHCG* expression negatively correlated with the infiltration of memory CD4+ and CD8+ T cells, natural killer cells, macrophages, dendritic cells, neutrophils and monocytes. By analyzing the correlation between *GIHCG* and immune cell markers, we found that increased *GIHCG* expression was significantly associated with the abovementioned immune cells. This finding suggests that *GIHCG* may play a role in regulating the microenvironment in HCC and influences HCC patient prognosis. This hypothesis and the detailed mechanism warrant further investigation.

Conclusion

In conclusion, our results provide evidence that *GIHCG* expression is increased in HCC and leads to a poor prognosis, making it possible for *GIHCG* to be considered a novel prognostic biomarker for HCC. In addition, *GIHCG* may affect the infiltration and function of immune cells in HCC.

Abbreviations

HCC, Hepatocellular carcinoma; lncRNAs, Long noncoding RNAs; *GIHCG*, lncRNA increased during hepatocarcinogenesis; GEPIA, Gene Expression Profiling Interactive Analysis; TCGA, The Cancer Genome Atlas; LIHC, Liver Hepatocellular Carcinoma; TPM, transcripts per kilobase million; OS, overall survival; DFS, disease-free survival; ANT, adjacent normal tissue; qRT-PCR, Quantitative real-time PCR; CISH, Chromogenic in situ hybridization; GSEA, Gene Set

Enrichment Analysis; TIICs, tumor-infiltrating immune cells; ssGSEA, single sample gene set enrichment analysis.

Acknowledgment

These authors contributed equally to the article and should be considered as co-first authors: Siyu Xiao and Shanzhou Huang.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68(6):394–424. doi:10.3322/caac.21492
2. Allemani C, Matsuda T, Di Carlo V, et al. Global surveillance of trends in cancer survival 2000–14 (CONCORD-3): analysis of individual records for 37 513 025 patients diagnosed with one of 18 cancers from 322 population-based registries in 71 countries. *Lancet*. 2018;391(10125):1023–1075. doi:10.1016/S0140-6736(17)33326-3
3. Tzartzeva K, Obi J, Rich NE, et al. Surveillance Imaging and Alpha Fetoprotein for Early Detection of Hepatocellular Carcinoma in Patients With Cirrhosis: A Meta-analysis. *Gastroenterology*. 2018;154(6):1706–1718. doi:10.1053/j.gastro.2018.01.064
4. Forner A, Reig M, Bruix J. Hepatocellular carcinoma. *Lancet*. 2018;391(10127):1301–1314.
5. Bruix J, Reig M, Evidence-Based Diagnosis SM. Staging, and Treatment of Patients With Hepatocellular Carcinoma. *Gastroenterology*. 2016;150(4):835–853.
6. Ponting CP, Oliver PL, Reik W. Evolution and functions of long noncoding RNAs. *Cell*. 2009;136(4):629–641.
7. Gugnoni M, Ciarrocchi A. Long Noncoding RNA and Epithelial Mesenchymal Transition in Cancer. *Int J Mol Sci*. 2019;20:8.
8. Zou Y, Xu S, Xiao Y, et al. Long noncoding RNA LERFS negatively regulates rheumatoid synovial aggression and proliferation. *J Clin Invest*. 2018;128(10):4510–4524.
9. Chen C, He W, Huang J, et al. LNMAT1 promotes lymphatic metastasis of bladder cancer via CCL2 dependent macrophage recruitment. *Nat Commun*. 2018;9(1):3826.
10. Fatica A, Bozzoni I. Long non-coding RNAs: new players in cell differentiation and development. *Nat Rev Genet*. 2014;15(1):7–21. doi:10.1038/nrg3606
11. Liang W-C, Ren J-L, Wong C-W, et al. LncRNA-NEF antagonized epithelial to mesenchymal transition and cancer metastasis via cis-regulating FOXA2 and inactivating Wnt/β-catenin signaling. *Oncogene*. 2018;37(11):1445–1456. doi:10.1038/s41388-017-0041-y
12. Yuan J-H, Yang F, Wang F, et al. A Long Noncoding RNA Activated by TGF-β Promotes the Invasion-Metastasis Cascade in Hepatocellular Carcinoma. *Cancer Cell*. 2014;25(5):666–681. doi:10.1016/j.ccr.2014.03.010
13. Sui C-J, Zhou Y-M, Shen W-F, et al. Long noncoding RNA *GIHCG* promotes hepatocellular carcinoma progression through epigenetically regulating miR-200b/a/429. *J Mol Med (Berl)*. 2016;94(11):1281–1296. doi:10.1007/s00109-016-1442-z
14. Zhang X, Mao L, Li L, He Z, Wang N, Song Y. Long noncoding RNA *GIHCG* functions as an oncogene and serves as a serum diagnostic biomarker for cervical cancer. *J Cancer*. 2019;10(3):672–681. doi:10.7150/jca.28525
15. He ZH, Qin XH, Zhang XL, Yi JW, Han JY. Long noncoding RNA *GIHCG* is a potential diagnostic and prognostic biomarker and therapeutic target for renal cell carcinoma. *Eur Rev Med Pharmacol Sci*. 2018;22(1):46–54.
16. Zhao H, Xing G, Wang Y, Luo Z, Liu G, Meng H. Long noncoding RNA HEIH promotes melanoma cell proliferation, migration and invasion via inhibition of miR-200b/a/429. *Biosci Rep*. 2017;37(3):3. doi:10.1042/BSR20170682
17. Jiang X, Li Q, Zhang S, Song C, Zheng P. <p>Long noncoding RNA *GIHCG* induces cancer progression and chemoresistance and indicates poor prognosis in colorectal cancer. *Onco Targets Ther*. 2019;12:1059–1070. doi:10.2147/OTT.S192290
18. Liu G, Jiang Z, Qiao M, Wang F. Lnc-*GIHCG* promotes cell proliferation and migration in gastric cancer through miR- 1281 adsorption. *Molecular Genetics & Genomic Medicine*. 2019;7(6):e711. doi:10.1002/mgg3.711
19. Yao N, Yu L, Zhu B, Gan HY, Guo BQ. LncRNA *GIHCG* promotes development of ovarian cancer by regulating microRNA-429. *Eur Rev Med Pharmacol Sci*. 2018;22(23):8127–8134.
20. Fan LY, Shi KY, Xu D, et al. LncRNA *GIHCG* regulates microRNA-1281 and promotes malignant progression of breast cancer. *Eur Rev Med Pharmacol Sci*. 2019;23(24):10842–10850.
21. Ma L, Wang Q, Gong Z, Xue L, Zuo Z. Long noncoding RNA *GIHCG* enhanced tongue squamous cell carcinoma progression through regulating miR-429. *J Cell Biochem*. 2018;119(11):9064–9071. doi:10.1002/jcb.27164
22. Huang S, Li J, Tam NL, et al. Uridine-cytidine kinase 2 upregulation predicts poor prognosis of hepatocellular carcinoma and is associated with cancer aggressiveness. *Mol Carcinog*. 2019;58(4):603–615. doi:10.1002/mc.22954
23. Huang S, Zhong W, Shi Z, et al. Overexpression of signal sequence receptor γ predicts poor survival in patients with hepatocellular carcinoma. *Hum Pathol*. 2018;81:47–54. doi:10.1016/j.humpath.2018.06.014
24. Colaprico A, Silva TC, Olsen C, et al. TCGAbiolinks: an R/Bioconductor package for integrative analysis of TCGA data. *Nucleic Acids Res*. 2016;44(8):e71. doi:10.1093/nar/gkv1507
25. Ritchie ME, Phipson B, Wu D, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*. 2015;43(7):e47. doi:10.1093/nar/gkv007
26. Yu G, Wang L-G, Han Y, He Q-Y. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS: A Journal of Integrative Biology*. 2012;16(5):284–287. doi:10.1089/omi.2011.0118
27. Hackl H, Charoentong P, Finotello F, Trajanoski Z. Computational genomics tools for dissecting tumour–immune cell interactions. *Nat Rev Genet*. 2016;17(8):441–458. doi:10.1038/nrg.2016.67
28. Charoentong P, Finotello F, Angelova M, et al. Pan-cancer Immunogenomic Analyses Reveal Genotype-Immunophenotype Relationships and Predictors of Response to Checkpoint Blockade. *Cell Rep*. 2017;18(1):248–262. doi:10.1016/j.celrep.2016.12.019
29. Jia Q, Wu W, Wang Y, et al. Local mutational diversity drives intratumoral immune heterogeneity in non-small cell lung cancer. *Nat Commun*. 2018;9(1):5361. doi:10.1038/s41467-018-07767-w
30. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A*. 2005;102(43):15545–15550. doi:10.1073/pnas.0506580102
31. Zhou J, Ding T, Pan W, Zhu L-Y, Li L, Zheng L. Increased intratumoral regulatory T cells are related to intratumoral macrophages and poor prognosis in hepatocellular carcinoma patients. *Int J Cancer*. 2009;125(7):1640–1648. doi:10.1002/ijc.24556
32. Dong P, Ma L, Liu L, et al. CD86+/CD206+, Diametrically Polarized Tumor-Associated Macrophages, Predict Hepatocellular Carcinoma Patient Prognosis. *Int J Mol Sci*. 2016;17(3):320. doi:10.3390/ijms17030320

33. Lu C, Rong D, Zhang B, et al. Current perspectives on the immunosuppressive tumor microenvironment in hepatocellular carcinoma: challenges and opportunities. *Mol Cancer*. 2019;18(1):130. doi:10.1186/s12943-019-1047-6
34. Fu Y, Liu S, Zeng S, Shen H. From bench to bed: the tumor immune microenvironment and current immunotherapeutic strategies for hepatocellular carcinoma. *J Exp Clin Cancer Res*. 2019;Volume 12 (1):396. doi:10.1186/s13046-019-1396-4
35. Kitagawa M, Kitagawa K, Kotake Y, Niida H, Ohhata T. Cell cycle regulation by long non-coding RNAs. *Cell Mol Life Sci*. 2013;70 (24):4785–4794.
36. Su M, Wang H, Wang W, et al. LncRNAs in DNA damage response and repair in cancer cells. *Acta Biochim Biophys Sin (Shanghai)*. 2018;50(5):433–439.
37. Iaccarino I. lncRNAs and MYC: an Intricate Relationship. *Int J Mol Sci*. 2017;18:7.
38. Cawley S, Bekiranov S, Ng HH, et al. Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. *Cell*. 2004;116(4):499–509.
39. Chen D, Lu T, Tan J, Li H, Wang Q, Wei L. Long Non-coding RNAs as Communicators and Mediators Between the Tumor Microenvironment and Cancer Cells. *Front Oncol*. 2019;9:739.
40. Luo Y, Yang J, Yu J, et al. Long Non-coding RNAs: emerging Roles in the Immunosuppressive Tumor Microenvironment. *Front Oncol*. 2020;10:48.
41. Ji J, Yin Y, Ju H, et al. Long non-coding RNA Lnc-Tim3 exacerbates CD8 T cell exhaustion via binding to Tim-3 and inducing nuclear translocation of Bat3 in HCC. *Cell Death Dis*. 2018;9(5):478.
42. Jiang R, Tang J, Chen Y, et al. The long noncoding RNA lnc-EGFR stimulates T-regulatory cells differentiation thus promoting hepatocellular carcinoma immune evasion. *Nat Commun*. 2017;8:15129.