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Original Article

Clinical diagnostic value of PIMREG on liver cancer cell phenotype and tumorigenic ability in nude mice

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Abstract: Objectives: In vitro experiments were manipulated to investigate the effect of the *PIMREG* (PICALM-interacting mitotic regulator gene) expression level on the malignant phenotype of liver cancer cells and their tumorigenesis ability in nude mice, and bioinformatics were used to analyze the clinical diagnostic and prognostic value in liver cancer. Methods: After liver cancer-related data were obtained from the TCGA database and GTEx database, the differences in *PIMREG* expression in liver cancer and normal liver tissue were compared using bioinformatics, and their correlation with the clinical pathological characteristics of liver cancer and the prognosis value were analyzed. A knockdown and overexpression model of *PIMREG* was constructed using Huh7 cells. The effect of the *PIMREG* expression level on the malignant phenotype of Huh7 cells was tested through CCK-8 and Transwell experiments. At the same time, animal knockdown and overexpression models were constructed to study the effect of the *PIMREG* expression level on the tumorigenesis ability in nude mice. Results: Bioinformatics analysis showed that *PIMREG* mRNA was significantly overexpressed in liver cancer tissue ($P<0.001$). There were differences in T-staging ($P<0.001$), pathological staging ($P=0.002$), vascular infiltration ($P<0.001$), histological grading ($P<0.001$), and AFP levels ($P<0.001$) between the high- and low-expression groups. A high expression of *PIMREG* is associated with a poor prognosis, manifested as a significant decrease in the overall survival, disease-specific survival, and progression-free survival rates of patients (P values of 0.006, 0.014, and 0.002, respectively). In the *PIMREG* overexpression model, the proliferation rate and invasion ability of Huh7 cells were significantly increased, and the tumorigenesis ability of nude mice was significantly enhanced. In the knockdown model, the opposite results were observed. Conclusions: The *PIMREG* gene is highly expressed in hepatocellular carcinoma, and increasing its expression level can significantly promote the malignant phenotype of liver cancer cells and their tumorigenesis ability in nude mice. Knocking down its expression level has the opposite effect. The expression level of *PIMREG* is related to the pathological stages of liver cancer patients, and its elevated expression is a risk factor for poor prognosis. *PIMREG* may become a new target for the clinical diagnosis, treatment, and prognosis evaluation of liver cancer.

Keywords: *PIMREG* expression, liver cancer, tumor formation in nude mice, staging, prognosis of liver cancer

Introduction

Liver cancer (LIHC) ranks as fourth most frequent among all cancers that lead to death worldwide, and the vulnerable population shows an increasing trend among youth [1]. According to the classification of histopathological examination, among the patient population, approximately 75%-80% of cases are attributed to hepatocellular carcinoma (HCC) with primary liver cancer, while intrahepatic cholangiocarcinoma (ICC) and other types of liver cancer account for 10%-15% of cases [2, 3]. The occurrence and development of LIHC are associated with various cellular signaling pathways, gene mutations, inflammatory damage, and abnormal vascular proliferation, which are complex and multistep processes [4, 5]. In recent years, a multitude of studies on transcription factors, mitotic regulators, and signaling pathway molecules associated with hepatocarcinogenesis have emerged. Nevertheless, the response and subsequent survival rates of patients with advanced liver cancer on existing molecular-targeted drugs are not as desirable as expected [6, 7]. Furthermore, molecular targets with satisfactory prognostic value have not yet been reported.

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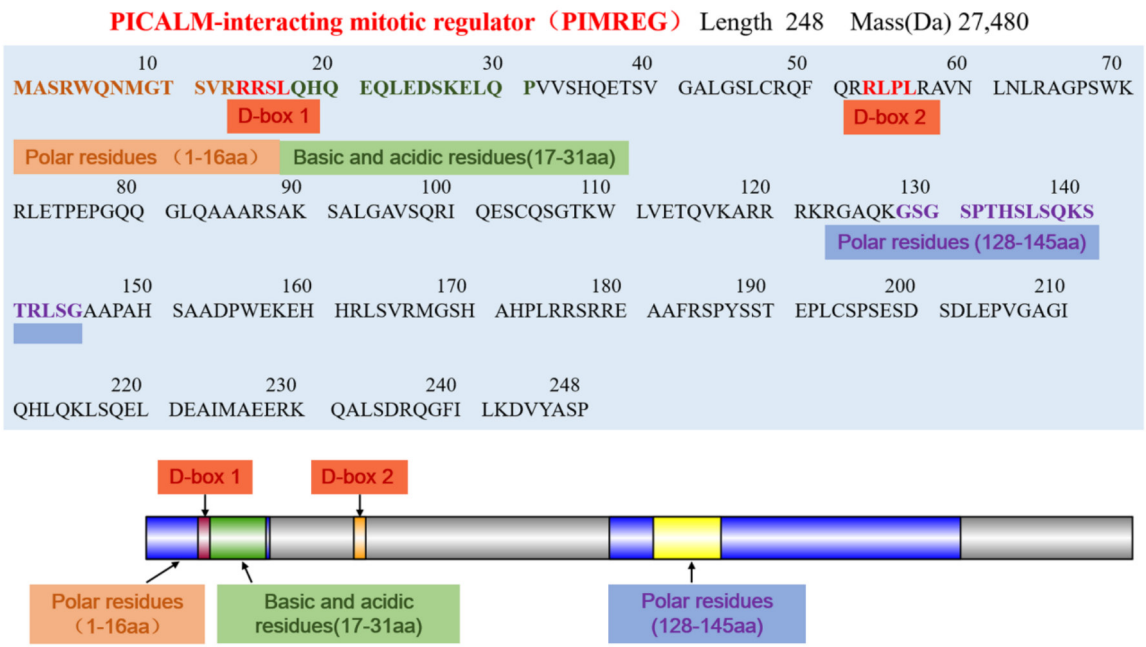

Figure 1. Domains of *PIMREG* (*PICALM-interacting mitotic regulator gene*).

PIMREG (PICALM-interacting mitotic regulator gene) is a mitotic regulator that interacts with the phosphatidylinositol and clathrin assembly protein (PICALM), also known as FAM64A (a family with a sequence similarity of 64; member A), CATS (cathepsin S), and RCS1 (RCSD domain containing 1), which has a total length of 248 amino acids and a molecular mass of 27,480 Daltons (**Figure 1**). *PIMREG* was first found to be expressed in the thymus, colon, and spleen, and was found to regulate cell proliferation [8-11]. Recently, based on the findings of most studies, we have discovered that, for most types of cancer, their occurrence and later development are directly related to *PIMREG*. Specifically, *PIMREG* not only influences the DNA repair process but also promotes cell cycle progression [12-14]. As has been recently reported, the molecular regulatory function of *PIMREG* is associated with the cleavage of mRNA, DNA repair, cell cycle regulation, Rho GTPase signal transduction, and the regulation of TP53 transcription and corresponding translation [15]. Simultaneously, *PIMREG* overexpression activates the NF kB (nuclear factor k-gene-binding) signaling pathway, increasing the invasiveness of breast cancer cells [13]. *PIMREG* overexpression stimulates gliomas by activating the β-catenin signaling pathway, and excessive *PIMREG* expression leads to glioma proliferation and glioma invasion [14]. Additionally, *PIMREG* is involved in the DNA damage response of glioblastoma cells and contributes to temozolomide resistance [16]. To date, few studies have explored the specific functions and clinical value of *PIMREG* in patients with LIHC. Based on this, we first explored the clinical value of *PIMREG* expression in LIHC using a combination of the GTEx and TCGA databases, before conducting in vitro cell experiments and animal tumorigenesis experiments to investigate the crucial role of *PIMREG* in the occurrence of LIHC.

Materials and methods

Bioinformatics data analysis and the clinical characteristics of and prognostic impact on liver cancer patients

We obtained detailed mRNA expression data from liver cancer patients using the TCGA database in conjunction with the GTEx database. The clinical and prognostic data of the patients were also downloaded, which consisted of 374 liver cancer tissue samples and 50 normal tissue samples, with 50 patients having matching adjacent normal liver tissue data. This study, as it involves human data, has undergone review and approval by the Medical College of Yangzhou Polytechnic University.

When explaining the clinicopathological characteristics of the patients, samples with missing or incomplete data and zero survival time were deleted. Subsequently, we divided the selected patients with liver cancer into the high- and low-expression groups (with 187 patients in each group). The low-expression group, comprising 187 cases, was established by utilizing the median expression level of *PIMREG* (0.132) in the liver cancer tissue samples. Gender, age, body mass index, TNM staging system classification (Stage N, Stage T, and Stage M), the stage of development of the patient's condition, and the histological grade of the cancer, as well as AFP, Alb, and the presence or absence of vascular infiltration, were selected as clinical and pathological characteristics of liver cancer. This part of the research aimed to explore the potential relationship between the clinical characteristics of liver cancer patients and their actual *PIMREG* gene expression levels. The main analytical methods used to test this were the Kruskal-Wallis test, the logical regression interpretation method, and the Wilcoxon rank-sum interpretation explanation method.

After combining the *PIMREG* gene expression level and patient survival follow-up data from the TCGA database, the Kaplan-Meiers estimator was employed to investigate the differences in progression-free survival, disease-specific survival, and total survival between the low- and high-expression groups. If the *P*-value obtained from the log-rank test was under 0.05, it implied statistical significance in the results. The primary objective of this section of the research was to explore the prognostic value of *PIMREG* expression in liver cancer. The main methods used for this included the multivariate Cox regression explanation method and the univariate Cox regression explanation method.

Establishment of PIMREG knockdown and overexpression cell sublines

The normal human liver cell L02 and liver cancer cell Huh7 were cell lines from the China Academy of Science, and the plasmid was pcDNA3.1 (Thermofly, USA). Firstly, Huh7 cells were cultured in DMEM substrate with CO_2, containing 10% FBS (Gibco, USA), and the temperature was set at 37°C and the humidity at 5%. Four sets of Huh7 cell models were constructed for subsequent experiments: the si-NC group (in which negative siRNA could be used to transfect object A), si-*PIMREG* group (which was transfected with *PIMREG* siRNA), vector group (in which a negative overexpression plasmid was used), and OE-*PIMREG* group (which was transfected with a *PIMREG* overexpression plasmid).

Real-time quantitative reverse transcription PCR (qRT-PCR) detection

First, total RNA was extracted from L02 cells, Huh7 cells, and four subtypes of Huh7 cells using TRIZOL reagent (Beyotime, MA, USA). Then, the extracted RNA was transcribed into cDNA for fluorescence quantitative real-time PCR detection. Subsequently, three holes were created in each sample type. Notably, the upstream and actual sequence expressions of the downstream primer corresponding to *PIMREG* were 5'-GTGCTTTGGGTGCCGTGTC-3' and 5'-ATCGCCGTAATGGGTGGG-3', respectively. The *PIMREG* amplification product was 268 bp in length. The reference for the internal implementation of GAPDH mainly referred to the upstream primer sequence and included the downstream primer sequence. The expression was as follows: 5'-GACAAGCTTCCCGTTCTCAG-3' and 5'-GAGTCAACGGATTTGGTGGT-3'. The primers used were obtained from the synthetic primers of China Sangong Biotechnology Technology Co., Ltd. The 2DDCT method was used to determine the relative transcription levels of *PIMREG*.

CCK-8 (cell counting kit-8) assay for cell viability

Cell proliferation tests were conducted on the four groups of Huh7 cells using a CCK8 kit (Solarb, China). Subsequently, the first step in this experiment was to adjust the actual concentration of cells, which was roughly (1-10) × 10^5 cells/ml, after which 100 μL cell suspensions were extracted and inoculated into 96-well plates for 48 h. Then, a solution was added to each well, and the actual dose for determining the OD450 and calculating the increment rate was 10 μL of CCK-8 solution.

Transwell assays for detecting cell invasiveness

Transwell assays were conducted on the four groups of Huh7 cells. For cells in the logarith-

mic phase, serum-free DMEM was generally used to dilute and adjust the concentration, and the actual concentration was (1-10) × 10^5 cells/mL. We transferred the LDMEM cell suspension to the chamber in the upper part of the Transwell carefully, using a volume of 200 μL. At the same time, a complete culture medium, supplemented with 10% FBS at a dosage of 700 μL, was put into the chamber in the lower part of the Transwell. After a whole day of training, it was first fixed with 4% formaldehyde for half an hour and then carefully stained with 1% crystal violet, before being placed under a 200 × microscope to capture images.

Establishment of PIMREG knockdown and overexpression mouse models

Male BALB/C nude mice, weighing 18-22 g (Beijing Weitong Lihua) and aged 4-5 weeks, were selected for adaptive feeding for one week. The selected nude mice were randomly divided into si-NC, si-*PIMREG*, vector, and OE-*PIMREG* groups. Subsequently, we injected four corresponding subtypes of Huh7 cells subcutaneously into the right flank of each mouse-the actual dose administered was 5 × 10^6 cells, with three mice in each group; we then returned the mice to the cage and monitored the increase in tumor size. Starting from the 4th week, we used Vernier calipers to measure the length and width of the mouse tumors once a week. The formula provided for calculating the actual tumor volume in each group was as follows:

$$\text{Tumor volume} = (\text{length} \times \text{Width}^2)/2 \quad (1)$$

The four groups of tumor tissues were photographed and weighed after eight weeks of feeding. Isoflurane inhalation method was used to euthanize the nude mice. The Ethics Committee on Experimental Animals of the Guangzhou Medical Experimental Animal Center granted their approval and consent for the management of this animal experiment.

Western blot analysis

We used protein imprinting to determine the *PIMREG* expression levels in four groups of Huh7 cell subtypes and four groups of mouse tumor tissues. The anti-*PIMREG* antibody (ab-162752, Abcam) was purchased from Abcam, and the secondary antibody was a goat anti-body against rabbit IgG H&L/HRP (Bioworld-bs-0295G-HRP). We used GAPDH (Abcam ab-181602) as the internal reference and performed Western blotting using conventional methods.

Statistical analysis

Bioinformatics research data were statistically explained using R 4.0.2 and SPSS 25.0. The nonparametric Mann-Whitney U test method was adopted, to investigate whether the *PIMREG* gene showed different expression levels for normal tissues versus liver cancer tissues. To explore whether different *PIMREG* expression levels in the two groups caused changes in clinical pathological features, we primarily utilized the Chi-squared test. In addition, prognostic explanations were provided through Kaplan-Meier survival analysis, Cox univariate analysis, and Cox multivariate analysis. When the *P* value was not greater than 0.05, the results in the above statistical results were considered obviously different and, therefore, statistically significant. To accurately evaluate the diagnostic value of differentially expressed genes, we utilized the "pROC package [version 1.17.0.1]" software package to generate receiver operating characteristic (ROC) curves, which represent subject characteristics of operation. To conduct a comprehensive series of statistical analyses, our research utilized the R environment (V3.6.3). The website http://www.r-project.org/ was employed for this purpose. We used the ggplot2 package in R [version 3.3.3] to create all the graphs. In vitro experimental data were analyzed using GraphPad principle 9.5.1. Intragroup data were descriptively explained using $\bar{x} \pm s$, and intergroup differences were evaluated using a one-way ANOVA. If there was a statistically significant difference, we conducted pairwise comparisons between groups using t-tests.

Results

PIMREG has a high expression in liver cancer tissue and other types of tumors

Explaining unpaired data by adopting the TCGA database illustrated that *PIMREG* expression levels were significantly elevated in liver cancer tissue samples, compared to normal tissue samples ($P<0.001$, **Figure 2A**). Using the TCGA-GTEx database for non-paired data, it was also found that *PIMREG* had a significantly higher

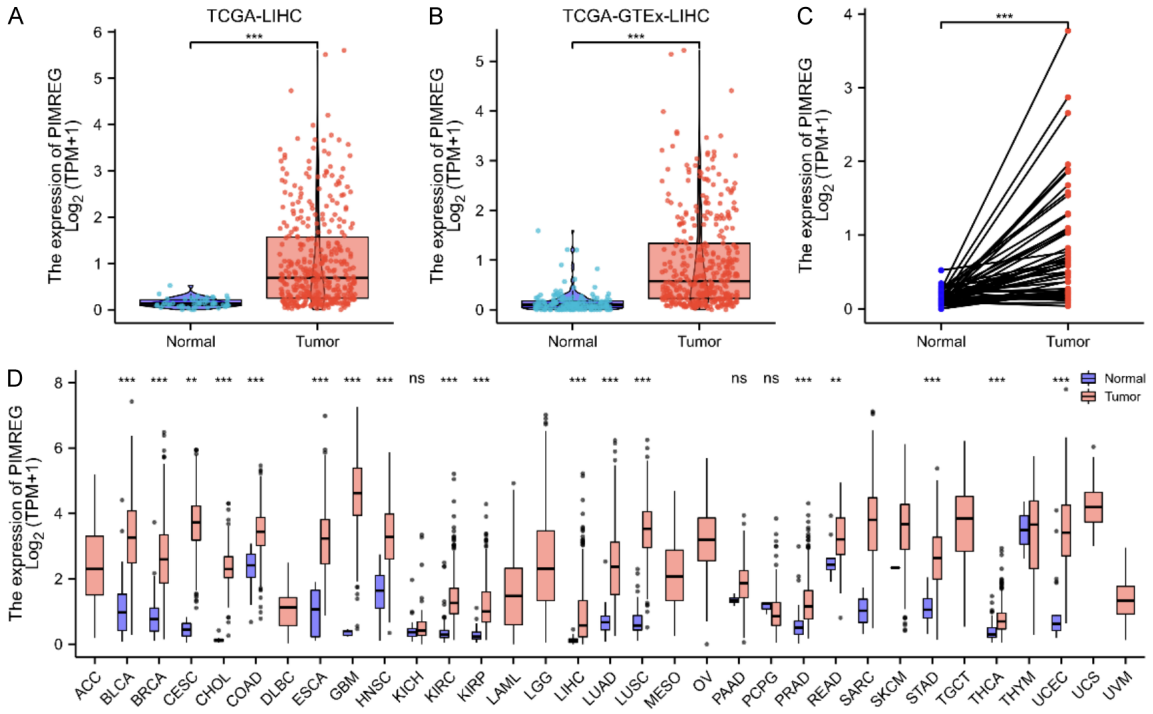

Figure 2. *PIMREG* (PICALM-interacting mitotic regulator gene) expression levels in various types of tumors and its relationship with liver cancer. A. Analysis of unpaired data from TCGA database. Compared to normal tissues, there was a significant upregulation of *PIMREG* in Hepatocellular carcinoma (HCC) tissues. B. Analysis of unpaired data from TCGA-GTEX composite database. Compared to normal tissues, *PIMREG* has a significant upregulation in HCC tissues. C. Analysis of paired data from TCGA database. Compared with normal tissues, HCC tissues exhibit a significant upregulation of *PIMREG*. D. Expression level analysis of *PIMREG* in different tumors and adjacent normal tissues. The expression of *PIMREG* is significantly upregulated in most tumor tissues.

expression in liver cancer tissue (P<0.001, **Figure 2B**). By collecting paired data from the tumor tissue and surrounding normal tissues for interpretation, consistent results were obtained (P<0.001, **Figure 2C**). Subsequently, we explored *PIMREG* expression levels in various cancers using the TCGA database. The *PIMREG* gene was upregulated in a majority of tumor tissues, inclusive of polymorphous glioblastoma, head and neck squamous cell carcinoma, and cholangiocarcinoma (**Figure 2D**).

Correlation between PIMREG and basic clinical information of LIHC patients

We screened 374 patients with liver cancer for their complete clinical data. Among them, the number of cases in the two groups was the same; that is to say, both the high- and low-expression group consisted of 187 cases each. *PIMREG* expression levels were significantly coordinated with T stage (P<0.001), pathological stage (P=0.002), BMI (P=0.004), sex (P< 0.001), age (P<0.001), histological grade (P< 0.001), AFP level (P<0.001), and vascular infiltration (P<0.001), but not with N stage, M stage, or Alb (**Table 1**). Subsequently, multiple comparisons were conducted to determine that, compared with T1, the expression of *PIMREG* was noticeably increased in T2/T3/T4 in the T stage (P<0.001). *PIMREG* expression was also remarkably upregulated in pathological stages I and II, compared to stages III and IV (P<0.05) (**Figure 3**).

The prognosis of liver cancer patients is closely interconnected with the upregulation of PIMREG

In the Kaplan-Meier analysis, it was observed that the upregulated *PIMREG* group had a lower overall survival rate (OS) (P=0.006), disease-specific survival rate (DSS) (P=0.014), and progression-free interval (PFI) (P=0.002) compared to the low *PIMREG* group (**Figure 4A-C**). According to the Cox regression analysis (**Figure 4A-C**), the *PIMREG* expression level was positively correlated with OS (HR=1.76), DSS (HR=

Table 1. Relationship between *PIMREG* and clinical information of patients

Characteristic	Downregulation of *PIMREG*	Upregulation of *PIMREG*	*P*
N	187	187	
T stage, n (%)			<0.001
T1	110 (29.6%)	73 (19.7%)	
T2	40 (10.8%)	55 (14.8%)	
T3	31 (8.4%)	49 (13.2%)	
T4	3 (0.8%)	10 (2.7%)	
N stage, n (%)			1.000
N0	119 (46.1%)	135 (52.3%)	
N1	2 (0.8%)	2 (0.8%)	
M stage, n (%)			0.128
M0	124 (45.6%)	144 (52.9%)	
M1	0 (0%)	4 (1.5%)	
Pathologic stage, n (%)			0.002
Stage I	102 (29.1%)	71 (20.3%)	
Stage II	37 (10.6%)	50 (14.3%)	
Stage III	32 (9.1%)	53 (15.1%)	
Stage IV	1 (0.3%)	4 (1.1%)	
BMI, n (%)			0.004
≤25	75 (22.3%)	102 (30.3%)	
>25	94 (27.9%)	66 (19.6%)	
Age, n (%)			<0.001
≤60	71 (19%)	106 (28.4%)	
>60	115 (30.8%)	81 (21.7%)	
Histologic grade, n (%)			<0.001
G1	41 (11.1%)	14 (3.8%)	
G2	93 (25.2%)	85 (23%)	
G3	50 (13.6%)	74 (20.1%)	
G4	0 (0%)	12 (3.3%)	
Alpha-Fetoprotein (ng/ml), n (%)			<0.001
≤400	129 (46.1%)	86 (30.7%)	
>400	17 (6.1%)	48 (17.1%)	
Albumin (g/dl), n (%)			0.733
<3.5	39 (13%)	30 (10%)	
≥3.5	123 (41%)	108 (36%)	
Vascular invasion, n (%)			<0.001
No	124 (39%)	84 (26.4%)	
Yes	43 (13.5%)	67 (21.1%)	
Gender, n (%)			<0.001
Female	45 (12%)	76 (20.3%)	
Male	142 (38%)	111 (29.7%)	
Age, median (IQR)	65 (56, 70)	59 (50.5, 67)	<0.001

1.87), and PFI (*HR*=1.65) (**Figure 4A-C**), suggesting that, in liver cancer, a high expression of *PIMREG* is strongly associated with a poor prognosis. As evidently exhibited by the ROC, the AUC value of *PIMREG* in liver cancer was 0.877, which demonstrates that *PIMREG* exhibits a strong correlation with the occurrence of liver cancer, making it a potential diagnostic marker for this disease (**Figure 4D**). Subgroup analysis demonstrated that a poor prognosis in

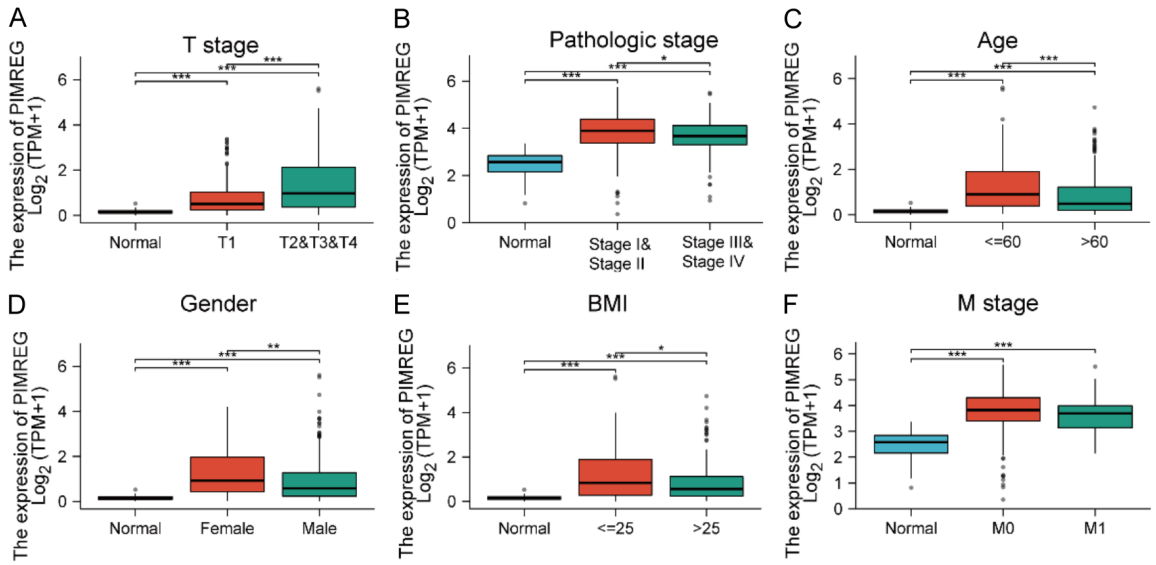

Figure 3. Correlation between clinicopathological features and *PIMREG* (PICALM-interacting mitotic regulator gene) expression level. A. Comparisons of T classification. Compared with T1 the expression of *PIMREG* was noticeably increased in T2/T3/T4. B. Evaluation of Pathologic stage. *PIMREG* gene's expression level in pathological stage I & II was strikingly higher than stage III & IV. C. Comparison of different Age groups. *PIMREG* gene's expression level in >60 subgroup was obviously lower than ≤60 subgroup. D. Gender analysis. *PIMREG* was remarkably higher in Female. E. Comparison of different BMI (Body Mass Index) groups. Compared with BMI ≤25 subgroup the expression of *PIMREG* was decreased in BMI <25 subgroup. F. Evaluation of M classification. No significant difference was come out in M-stage assay. **P*<0.05, ***P*<0.01, ****P*<0.001.

male patients was associated with a high expression of *PIMREG* (**Figure 4G**) and elderly patients (**Figure 4E**) (*P*<0.05), while the correlation was not significant in females (**Figure 4H**) or for patients under 65 years of age (**Figure 4F**) (*P*>0.05).

The univariate Cox regression assessment showed that *PIMREG* gene expression levels (*HR*=1.264, 95% *CI*: 1.081-1.478, *P*=0.003), M stage (*HR*=4.077, 95% *CI*: 1.281-12.973, *P*=0.017), T stage (*HR*=2.949, 95% *CI*: 1.982-4.386, *P*<0.001), and pathological stage (*HR*=2.504, 95% *CI*: 1.727-3.631, *P*<0.001) were all significant prognostic factors (**Table 2**). No independent risk factors were found after conducting a multivariate Cox regression analysis. However, among all evaluated factors, the expression of *PIMREG* exhibited the closest correlation with OS (*HR*=1.194, 95% *CI*: 0.989-1.443, *P*=0.066) (**Table 2**). In patients diagnosed with LIHC, a high *PIMREG* expression has been demonstrated to be a dependable predictor of an unfavorable prognosis when compared to other clinical indicators.

We conducted a study to examine whether there was a correlation between *PIMREG* expression and OS in subgroups based on age, sex, and T staging. In our findings, the upregulation of *PIMREG* was found to be correlated with adverse events in different subgroups. Specifically, these subgroups included individuals who were males (*P*=0.042) aged ≥65 years (*P*=0.001), as well as those in T1 and T2 stages (*P*=0.01) and T3 and T4 stages (*P*=0.028).

PIMREG is upregulated in LIHC

In this study, qRT-PCR was carried out to examine the difference in *PIMREG* expression between normal tissues extracted from the liver and liver cancer cells. As demonstrated by the experimental results, there was a strikingly higher level of *PIMREG* expression in Huh7 cells compared to L02 cells (*P*<0.01), indicating a high expression of *PIMREG* at the RNA level in liver cancer patients (**Figure 5**).

Successful establishment of the overexpression and knockdown models of PIMREG

To investigate the impact of the *PIMREG* expression level on the vicious expression type of liver cancer, we constructed liver cancer models with a high expression of *PIMREG* and a low

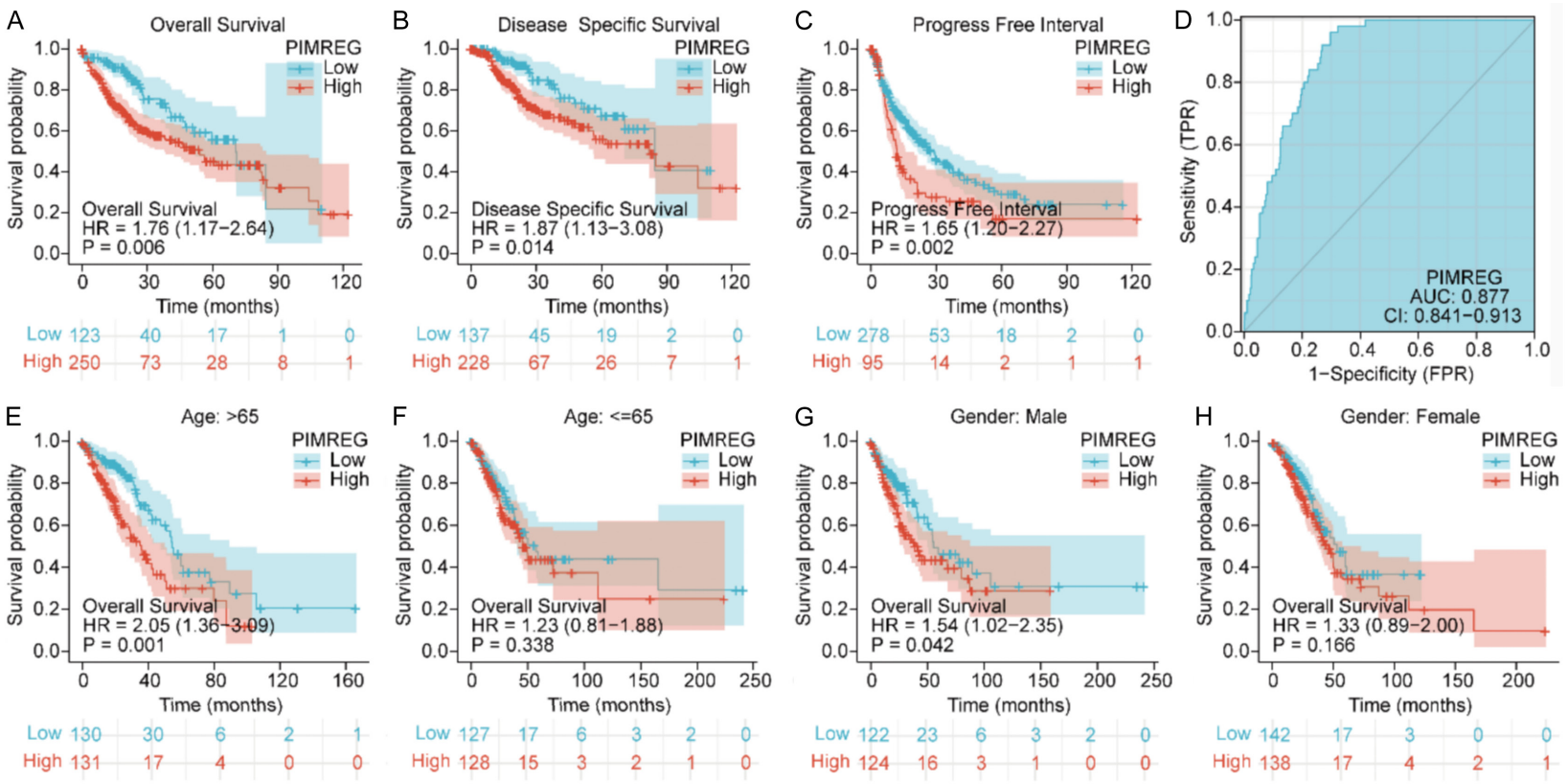

Figure 4. Connection between PIMERG expression and prognosis of Hepatocellular carcinoma (HCC). A. Overall survival (OS) assay. In the *PIMREG* upregulation group the OS time was obviously lower. B. Disease specific survival (DSS) analysis. In the upregulation group the DSS time was noticeably lower. C. Progression-free interval (PFI) assay. In the *PIMREG* upregulation group the PFI time was remarkably lower. D. ROC assay. The AUC value of *PIMREG* in liver cancer was 0.877. E. Correlation between *PIMREG* and OS in age >65 subgroup. In the elderly patients subgroup *PIMREG* was significantly associated with poor prognosis. F. Correlation between *PIMREG* and OS in age ≤65 subgroup. There was no significant correlation. G. Correlation between *PIMREG* and OS in male patients. In males, a high expression of *PIMREG* was linked to an unfavorable prognosis. H. Correlation between *PIMREG* and OS in female patients. The correlation was not significant.

Table 2. Multivariate and univariate assessment of the interconnection between clinicopathological characteristics and overall survival rate (OS) in Hepatocellular carcinoma (HCC)

Characteristics	Total (N)	Univariate explains		Multivariate explains	
		Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
T stage	370				
T1	183	Reference			
T2	94	1.428 (0.901-2.264)	0.129	1.423 (0.782-2.587)	0.248
T3 & T4	93	2.949 (1.982-4.386)	<0.001	1.728 (0.226-13.186)	0.598
M stage	272				
M0	268	Reference			
M1	4	4.077 (1.281-12.973)	0.017	2.180 (0.666-7.134)	0.197
Pathologic stage	349				
Stage I & Stage II	259	Reference			
Stage III & Stage IV	90	2.504 (1.727-3.631)	<0.001	1.732 (0.237-12.685)	0.589
PIMREG	373	1.264 (1.081-1.478)	0.003	1.194 (0.989-1.443)	0.066

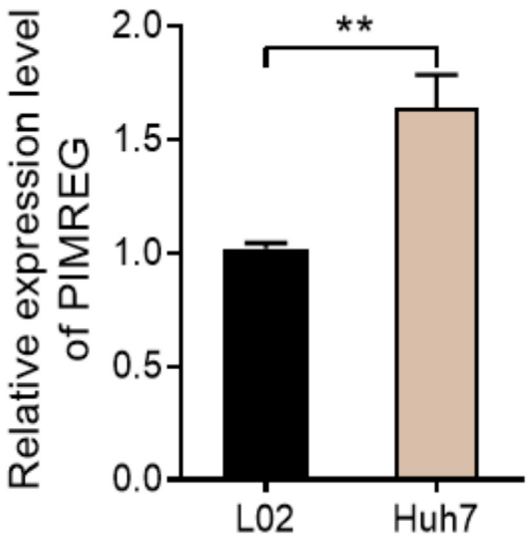

Figure 5. Real-time quantitative reverse transcription PCR (qRT-PCR) was utilized to confirm the upregulation of *PIMREG* in Huh7 cells. Compared with L02, Huh7 cells exhibit a significant upregulation of *PIMREG*. ***P*<0.01.

expression of *PIMREG*. Four sets of Huh7 cell sublines constructed in vitro were evaluated using qRT-PCR and Western blotting. As exhibited by those experimental findings, compared with the si-NC group, we found that the *PIMREG* expression level was markedly reduced in si-*PIMREG* group cells (*P*<0.01), but the opposite results were observed for the OE-*PIMREG* group, when compared to the vector group (*P*<0.01). We contrasted two control groups, the si-NC group and the vector group, to assess any potential non-specific effects on *PIMREG* expression (**Figure 6A**, **6B**). Four groups of mouse models were established in vitro, using the constructed cell models. Nude mice were cultured for eight weeks and the tumor tissue sizes were measured weekly. To determine the protein expression levels of *PIMREG* across different tissues, we employed Western blot (WB) analysis. The results showed that, compared to those in their respective control groups, *PIMREG* expression levels were significantly reduced in the si-*PIMREG* group (P< 0.01) and significantly increased in the OE-*PIMREG* group (P<0.01). However, our appraisal found no statistically significant differences between the two control groups (**Figure 6B**, **6C**). These results demonstrate that tumor formation in a nude mouse model was successfully conducted.

The effect of PIMREG expression on the invasion abilities and proliferation of LIHC cells

To examine the influence of *PIMREG* expression on the proliferation of LIHC (liver hepatocellular carcinoma) cells, we employed the CCK-8 assay. As evidently demonstrated by the results, in the si-*PIMREG* group, where *PIMREG* expression was knocked down, we observed a lower cell proliferation rate than in the si-NC group (*P*<0.01). Conversely, in the OE-*PIMREG* group, characterized by *PIMREG* overexpression, we found a higher cell proliferation rate than in the vector group (*P*<0.01). No significant differences were found when comparing the two control groups (**Figure 7A**). A high level of *PIMREG* expression promoted the proliferation of LIHC cells. However, low *PIMREG* expression levels inhibited the proliferation of LIHC cells.

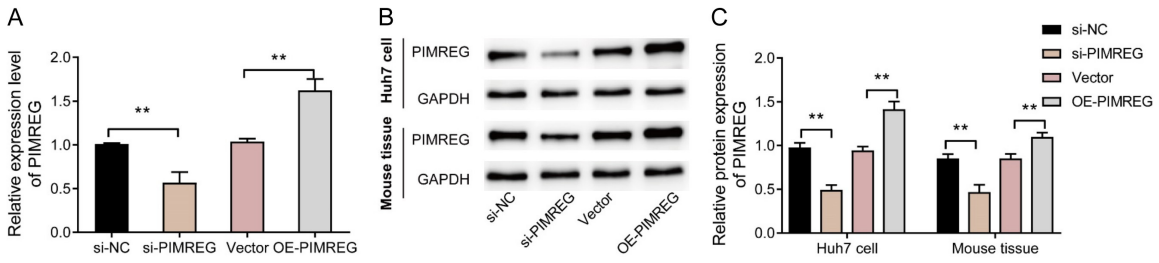

Figure 6. Hepatocellular carcinoma (HCC) models with upregulation and downregulation of *PIMREG* were constructed confirmed by in vitro experiments. (A) Real-time quantitative reverse transcription PCR (qRT-PCR). In the si-*PIMREG* group the *PIMREG* expression level was noticeably reduced compared with the si-NC group. In the OE-*PIMREG* group, there is a noticeable increase in *PIMREG* expression compared to the Vector group. (B, C) Western blot assays. *PIMREG* knockdown (si-*PIMREG* group vs si-NC group, upper in B and left in C) and over expression (OE-*PIMREG* group vs Vector group, bottom in B and right in C) was confirmed in Huh7 cell subline and mice transplanted tumors. ***P*<0.01.

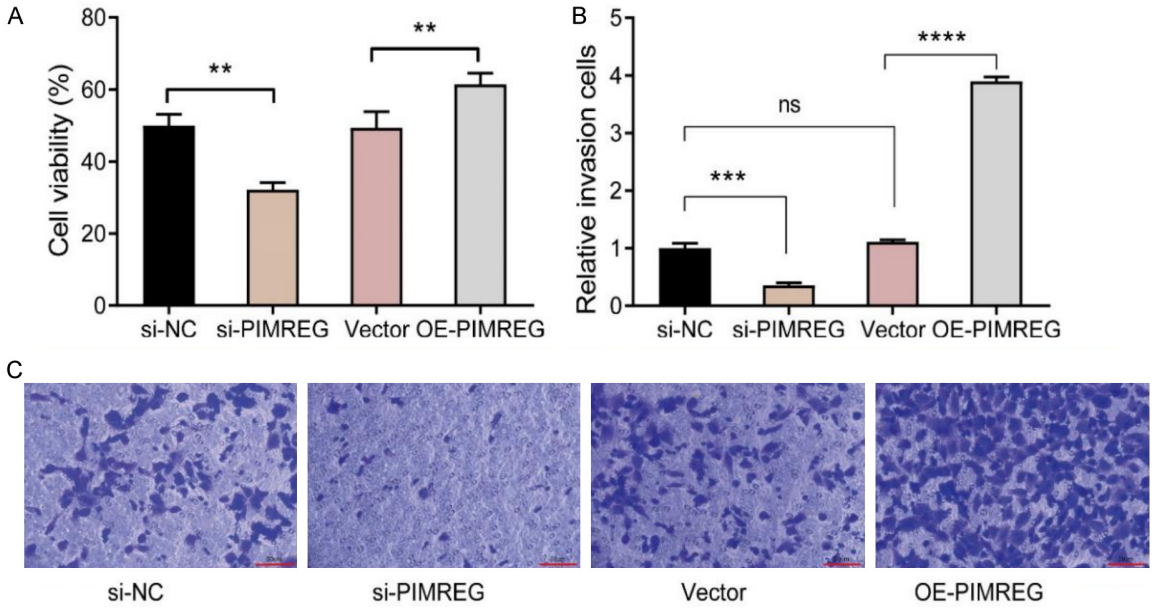

Figure 7. Validation of *PIMREG*'s ability to promote the aggressive phenotype of liver cancer cells. A. The viability of Huh 7 cells was assessed in four different groups using the CCK-8 (Cell Counting Kit-8) assay. In comparison to the si-NC group, the si-*PIMREG* group exhibited a lower cell proliferation rate. Conversely, the OE-*PIMREG* group displayed a higher cell proliferation rate compared to the Vector group. B, C. Invasion of Huh 7 cells from 4 groups was determined using Transwell assay. The cell's invasion ability of the si-*PIMREG* group was conspicuously reduced in comparison with the si-NC group, while the OE-*PIMREG* group displayed a noticeable increase in invasion compared to Vector group. 200 × microscope. ***P*<0.01, ****P*<0.001, *****P*<0.0001, ns means no statistically significant. Bar =50 μm.

Subsequently, to reveal the effect of *PIMREG* expression on the invasive capacity of LIHC cells, a Transwell assay was performed. The results obtained showed that the cells' invasion ability, in the *PIMREG* knockdown group, was significantly reduced, in comparison with the control group (si-NC group). Meanwhile, compared with the control group (the vector group), the invasive ability of the cells in the *PIMREG* overexpression group was noticeably upregulated, with highly significant differences found (*P*<0.0001) (**Figure 7B**, **7C**). These results indicate that promoting the expression of *PIMREG* can strengthen the invasive ability of LIHC cells, while inhibiting its expression can weaken it.

The influence of PIMREG expression on the tumorigenesis ability of LIHC cells in nude mice

The tumor formation rates of the four sets of nude mice with liver cancer constructed in this

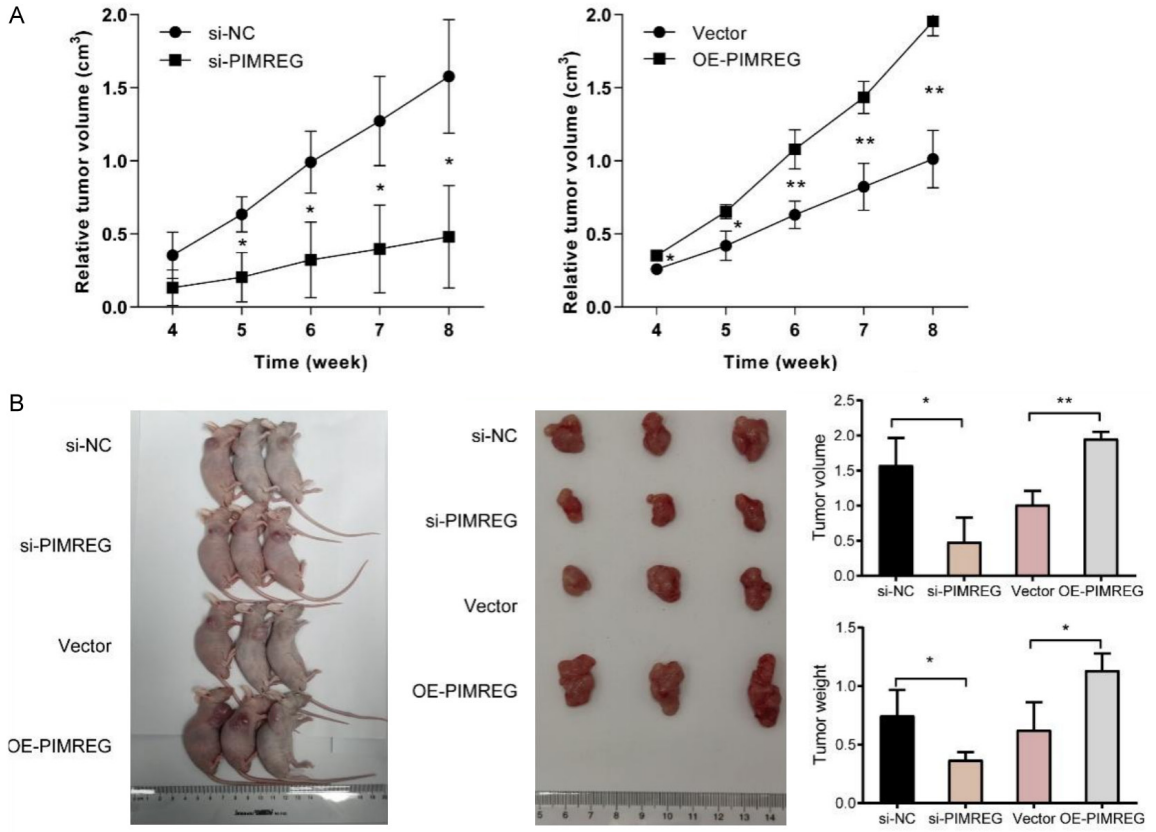

Figure 8. Validation of *PIMREG*'s ability to promote Tumorigenic of Nude Mice. A. Analysis of changes in tumor volume in 4 groups of nude mice during the 4-8 week period. From week 5 to week 8, the tumor volume was remarkable shrunk in si-*PIMREG* group compared to si-NC group (left side). Conversely, the tumor volume was notably enhanced in OE-*PIMREG* group when compared to Vector group (right side), this difference was significant from the 6th week onwards. B. Analysis of tumor volume and mass in 4 groups of nude mice in the 8 week. Four groups of nude mice were photographed (left). The measurements of the mouse tumors included their length and width (middle). As compared to the control group respectively, both volume and weight of tumor in si-*PIMREG* group were smaller while in the OE-*PIMREG* group were larger (right). **P*<0.05, ***P*<0.01.

study reached 100%, and the tumor volumes showed a continuous growth trend. As illustrated by our findings, from week 5 to week 8, the tumor volumes were found to be markedly lower in the inhibitory group compared to the control group ($P<0.05$) (**Figure 8A**, left). Conversely, the tumor volumes in the overexpression group were observed to be significantly higher ($P<0.05$), and this difference was significant from the 6th week onwards ($P<0.01$) (**Figure 8A**, right). At the end of the 8th week, the nude mice were euthanized, and the tumor volumes were assessed by weighing and calculating. The tumor weights of the *PIMREG* inhibition group and the control group were (0.370±0.066) and (0.750±0.217) g, respectively, with volumes of (0.481±0.202) and (1.577±0.388) cm^3. In the *PIMREG* inhibition group, both tumor volume and weight were found to be lower in comparison to the control group ($t=3.633$, $P=0.0221$; $t=2.909$, $P=0.0437$, respectively). The tumor weights in the *PIMREG* overexpression and control groups were (1.137±0.142) and (0.627±0.235) g, respectively, with volumes of (1.955±0.098) and (1.013±0.197) cm^3. Compared to the control group, both tumor volume and weight in the *PIMREG* overexpression group were larger ($t=7.402$, $P=0.0018$; $t=3.220$, $P=0.0323$, respectively) (**Figure 8B**).

Discussion

Through bioinformatics analysis, we first concluded that the *PIMREG* gene can show a high level of expression in LIHC compared to normal liver cells and the surrounding normal tissues, and that the overexpression of *PIMREG* is a sig-

nificant prognostic factor for OS, DSS, and PFI reduction. As the ROC curve clearly shows, during the process of diagnosing LIHC, one of the most remarkable biomarkers is *PIMREG*. Additionally, both univariate or multivariate Cox analyses proved that "*PIMREG* mRNA expression level", "T stage", "pathological stage", and "whether metastasis is present" can be used as prognostic indicators of LIHC, but none of the above function as independent prognostic indicators of LICH. Furthermore, we observed that the *PIMREG* expression level was remarkably higher in patients diagnosed as being at the T2, T3, and T4 stages compared to those at the T1 stage. Additionally, regarding the pathological stages, patients classified as stages I and II exhibited higher levels of *PIMREG* expression, suggesting a potential connection between elevated *PIMREG* expression and the extent of tumor vascular infiltration in LIHC. We also found higher levels of *PIMREG* expression in women and patients under 60 years of age, which is consistent with previous studies conducted on the same subject, indicating physiological differences in *PIMREG* expression [17]. In line with relevant reports, the probability of incidence of liver cancer in men (the ratio of male to female is 2.3-1) and the mortality rate are much higher than those in women [18]. The subgroup analysis performed in this research further confirmed that *PIMREG* upregulation was found to be connected with a poor prognosis in male and also elderly patients. However, this correlation was not significant in females and patients under 65 years of age. This suggests that *PIMREG* may become a brand-new objective biomarker for the diagnosis and classification of LIHC. However, physiological factors should be fully considered when *PIMREG* is applied in clinical practice.

In glioma, a high level of *PIMREG* expression can influence DNA damage repair and cell cycle regulation by upregulating RAD51, BRCA1, CDC25A, CDC25B, and CDC25C, or by downregulating HIPK2. Its overexpression can promote the cell cycle transition of glioma cells from G1 to the S phase [14, 16]. A high level of *PIMREG* expression promotes the migration and proliferation of tumor cells. For instance, its overexpression can remarkably enhance the proliferation of glioma cells and reinforce their migration and invasion abilities [16]. We conducted in vitro experiments on liver cancer with *PIMREG*, and the results supported previous findings that the overexpression of *PIMREG* in liver cancer enhanced both proliferation and invasion. This was further confirmed by knocking out *PIMREG*, which had inhibitory effects on the growth and invasiveness of LIHC cells. The impact of *PIMREG* knockdown on the aggressive phenotype of liver cancer is consistent with previous research reports [19].

We established a nude mouse tumor formation model under the overexpression and knockdown levels of *PIMREG* to investigate its impact on the occurrence of liver cancer, and confirmed that the overexpression of *PIMREG* continuously and significantly promoted the growth of liver cancer tumors, in terms of volume and mass, while knocking down the *PIMREG* expression level remarkably inhibited the tumorigenic ability of liver cancer.

Previous studies have focused on the effect of *PIMREG* in various kinds of cancer, such as lung cancer, renal cell carcinoma, pancreatic cancer, and breast cancer. Studies focusing on these cancer patients have highlighted the connection between altered *PIMREG* expression and its prognosis value [20-22]. However, no study targeting liver cancer patients has yet considered the prognostic significance of *PIMREG*. As evidenced by the TCGA database, we concluded that, in LIHC, there is a significant association between *PIMREG* upregulation and reduced overall survival. In addition, multiple comparisons have shown that a high *PIMREG* expression, as consistently observed across various subgroups of LIHC patients classified as being in the T1, T2, T3, and T4 phases, can continue to trigger poor survival, illustrating that *PIMREG* may also become a molecular marker for the prognosis of liver cancer patients.

We first compiled data from the GTEx and TCGA databases. Our analysis revealed that *PIMREG* upregulation was significantly correlated with an increased risk of LIHC occurrence, an advanced pathological stage, and an unfavorable prognosis for LIHC patients. Subsequently, to validate our findings, in vitro experiments were conducted. These experiments aimed to confirm the role of the upregulation of *PIMREG* in liver cancer. Interestingly, our results clearly demonstrated that an elevated *PIMREG* expression significantly enhanced the malignant characteristics of liver cancer cells, leading to

the noticeable promotion of cell proliferation. Conversely, suppressing its expression led to the opposite results. These findings suggest that, in liver cancer, *PIMREG* could potentially serve as a novel target for both diagnosis and treatment. Furthermore, its expression levels may hold prognostic value in assessing the progression and outcome of liver cancer.

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Disclosure of conflict of interest

None.

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