ORIGINAL RESEARCH

MARCKS is a New Prognostic Biomarker in Hepatocellular Carcinoma

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Background: Hepatocellular carcinoma (HCC) is one of the most common type of cancers, but there is still a lack of known biomarkers for the effective diagnosis or prognosis of HCC. Myristoylated alanine-rich C-kinase substrate (MARCKS) is a substrate of protein kinase C, which was located in the cell plasma membrane. The purpose of our study was to evaluate the role of MARCKS in HCC.

Methods: The role of MARCKS in HCC was explored by bioinformatics and experiment.

Results: We demonstrated that MARCKS expression was significantly elevated in HCC datasets of TCGA. MARCKS was up-regulated in tumor sample in HCC. Functional enrichment indicated that MARCKS-related differentially expressed genes (DEGs) were mainly enriched in cell junction tissue, response to growth factors and cell population proliferation. Tumor and ECM-receptor interactions related pathways were enriched by the KEGG. MARCKS expression in HCC patients was higher in females, younger individuals, and those at worse clinical stages. Cox regression analysis showed that MARCKS expression was a risk factor for overall survival and disease-specific survival of patients.

Conclusion: MARCKS was up-regulated in HCC, may play a crucial role in HCCs, and has prognostic value for clinical outcomes.

Keywords: myristoylated alanine-rich C-kinase substrate, HCC, TCGA, prognostic value

Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer, accounting for approximately 90% of all primary liver cancers.[1,2] The morbidity and mortality of HCC are on the rise worldwide, and it is the fourth leading cause of cancer-related death around the world.[3,4] Since HCC patients are usually in the advanced stage when first diagnosed and their tumors develop rapidly, only 5–15% of patients are eligible for surgical treatment. Despite advances toward early diagnosis and multidisciplinary treatment of cancer, the long-term prognosis of HCC patients remains poor. Therefore, it is necessary to identify new molecular markers for the early diagnosis of HCC. Currently, commonly used marker genes known marker genes for diagnosis of HCC include AFP,[5] AFP-L3[6] and Des-γ-carboxy prothrombin.[7] In addition, novel markers including ring finger related genes,[8] PSME4,[9] CCT7,[10] LINC01234,[11] PPP1R26,[12] and miR-10b-5p[13] in exosomes have also been demonstrated for diagnosis of HCC.

Myristoylated alanine-rich C-kinase substrate (*MARCKS*) is a member of a ubiquitous, highly conserved membrane-associated protein family. Wu et al discovered *MARCKS* served as a substrate for calcium/phospholipid-dependent phosphorylation of PKC in 1982 and first described it as the "87k protein" in the nerve endings of the rat cerebral cortex.[14] *MARCKS* is involved in the structural regulation of the actin cytoskeleton, cell motility, adhesion, etc.[15]

MARCKS was up-regulated several cancers, such as ovarian,[16] breast[17] and lung cancer.[18] Recent findings suggested that phospho-*MARCKS* is a novel NF-kB activator that promotes smoke-mediated lung cancer progression.[18] *MARCKS* promotes drug resistance in multiple myeloma and pancreatic cancer cells.[19,20] However, *MARCKS* has also been shown to inhibit tumor development. Eustace et al demonstrated that *MARCKS* ED peptide therapeutics may overcome traditional GBM resistance mechanisms. *MARCKS* is overexpressed in HCC,[21] and *MARCKS* on tumor-associated fibroblasts (TAMs) is related to poor HCC prognosis and immune cell infiltration.[22] This suggests that *MARCKS* expression may play a role in HCC tumorigenesis. However, the potential role and underlying mechanism of *MARCKS* expression in HCC remain unclear.

In this study, we explored that *MARCKS* expression was in HCC tissues than in adjacent normal tissues by analyzing data from HCC patients in TCGA and validated the results in our samples. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses showed the possible mechanism of *MARCKS* expression in the occurrence and development of HCC. We also analyzed the correlation between *MARCKS* expression and clinico-pathological characteristics of HCC. Finally, the diagnostic and prognostic value of *MARCKS* expression in HCC were determined.

Materials and Methods
Data Collection
MARCKS mRNA expression and clinical data from 374 LIHC and 50 normal control samples were obtained from TCGA-LIHC and cBioportal for Cancer Genomics.[23,24]

Human Hepatocellular Carcinoma Clinical Specimens and Ethics Statement
Primary hepatocellular carcinoma cancer tissues and pairs of adjacent non-cancerous tissues (ie, tissues more than 1 cm from the original site) were collected from hepatocellular carcinoma patients undergoing surgery in the Affiliated Hospital of Jining Medical University. We obtained 8 matched patient tissue specimens. All cancer tissue samples were confirmed by histopathology to have hepatocellular carcinoma. Figure S1 showed a representative pathological picture of liver cancer. Figure S2 showed the immunohistochemistry of *MARCKS* from Human Protein Atlas. The study complies with the principles outlined in the Declaration of Helsinki and was conducted in accordance with approved guidelines. Before collecting tissue samples, written informed consent was obtained from each participant. All samples were obtained in accordance with the regulations and approval of the Medical Ethics Committee of Affiliated Hospital of Jining Medical University (Ethical approval number: 2022C217).

RNA Extraction and Real-Time RT-PCR (mRNA)
RNA isolater (Vazyme Biotech co., Ltd.) was used to extract total RNA from cells and tissues. Real-time RT-PCR was employed to determine the expression of *MARCKS*. The cDNAs were synthesized according to the manufacturer's instructions (Vazyme Biotech co., Ltd.). Real-time quantitative RT-PCR (qRT-PCR) was conducted with the ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech co., Ltd). β-actin was used as an internal control. The primers were as following: β-actin (Forward- GACCTGTACGCCAACACAGT; Reverse- CTCAGGAGGAGCA ATGATCT), *MARCKS* (Forward- CCAACCCGAGGCTCTTTGTT; Reverse- CACGTGGCCAT TCTCCTGTC).

Differentially Expressed Genes of *MARCKS*
The 347 patients were divided into high and low *MARCKS* expression groups according to their median *MARCKS* values. The R package "DESeq2" was applied to calculate the differentially expressed genes between the two groups (FC >1.5, P value <0.05).[25] The "pheatmap" and "EnhancedVolcano" R packages were used to draw heatmaps and volcano maps of differentially expressed genes, respectively.

Functional Annotation of *MARCKS*-Associated Differentially Expressed Genes in HCC

The DEGs were functionally annotated in the Metascape database (count >3, enrichment factor >1.5, adjusted P < 0.01).[26] DEG gene set enrichment analysis (GSEA)[27] was performed by the R package "cluster Profiler"[28] The reference gene set was a selected gene set of the MSigDB collection, with a total of 796 clusters (FDR <0.25, P < 0.05). STRING[29] was used to analyse DEGs to obtain a protein–protein interaction (PPI) network, which is visualized using Cytoscape[30] (v3.7.1).

Association of *MARCKS* and Immune Cell Infiltration in HCC

The R package "GSVA"[31] was used to analyse the infiltration and enrichment of 24 common immune cells and calculate the Spearman coefficient to evaluate the relationship between *MARCKS* expression and immune cell infiltration. The Wilcoxon rank sum test was used to compare the level of immune cell infiltration in the high *MARCKS* expression group and the low *MARCKS* expression group.

Analysis of the Correlation Between *MARCKS* Expression and Clinicopathological Characteristics of HCC Patients

Association of clinicopathological features with *MARCKS* expression was analyzed by the Wilcoxon rank-sum test and the Pearson chi-square test. Correlation between *MARCKS* expression and the clinicopathological features was analyzed by Logistic regression.

Clinical Significance of *MARCKS* Expression in HCC

ROC was used to analyze the predictive value of *MARCKS* for the diagnosis of HCC. Prognostic analysis was performed by using univariate Kaplan–Meier (K-M) analysis and multivariate Cox regression analysis. Random forest regression was analyzed by the R package "randomForest".[32] Line graphs and calibration graphs was created by R packages "rms".[33] The R package "forestplot" was used to analyze the clinicopathological subgroups.[34] p < 0.05 was considered to be significant.

Results

Expression Profiles of *MARCKS* in Different Cancers and *MARCKS* Related Differentially Expressed Genes in Hepatocellular Carcinomas

We detected the expression of *MARCKS* in tumours and adjacent normal tissues in TCGA. As shown in Figure 1A, *MARCKS* was significantly overexpressed in 31 cancers, indicating that *MARCKS* is generally highly expressed in tumour tissues. More specifically, the expression of *MARCKS* in hepatocellular carcinoma was significantly higher than that in adjacent normal tissues (P < 0.001, Figure 1B). The overexpression of *MARCKS* was also verified in HCC patient tissues (Figure 1C).

According to the median *MARCKS* expression in HCC, 374 patients were divided into two groups, high- and low-*MARCKS* expression groups. We calculated differences in mRNA expression between the two groups. A total of 1013 mRNAs were identified (821 upregulated and 192 downregulated with HCC, Figure 1C) as DEGs (differentially expressed genes). The represented DEGs are presented with heatmaps (Figure 1D). The represented DEGs are presented with heatmaps (Figure 1E).

Functional Annotation of *MARCKS*-Associated DEGs in Hepatocellular Carcinomas

"Metascape" was used to explore the functions of *MARCKS*-related DEGs in HCC patients. As shown in Figure 2A–C, we found that DEGs were enriched in several hepatocellular carcinoma-related functions, including cell junction organization (GO: 0034330, P < 0.001, enrichment factor = 2.5, FDR = 0.037), response to growth factor (GO: 0070848, P < 0.001, enrichment factor = 2.3, FDR = 0.141), and cell population proliferation (GO: 0008285, P < 0.001, enrichment factor = 2.2, FDR = 0.266). In addition, GSEA showed that *MARCKS*-associated DEGs were significantly enriched in tumour-related pathways (Figures 2D–K), such as the PI3K-AKT signalling pathway (P.adjusted = 0.040, FDR = 0.031), Hippo-Merlin
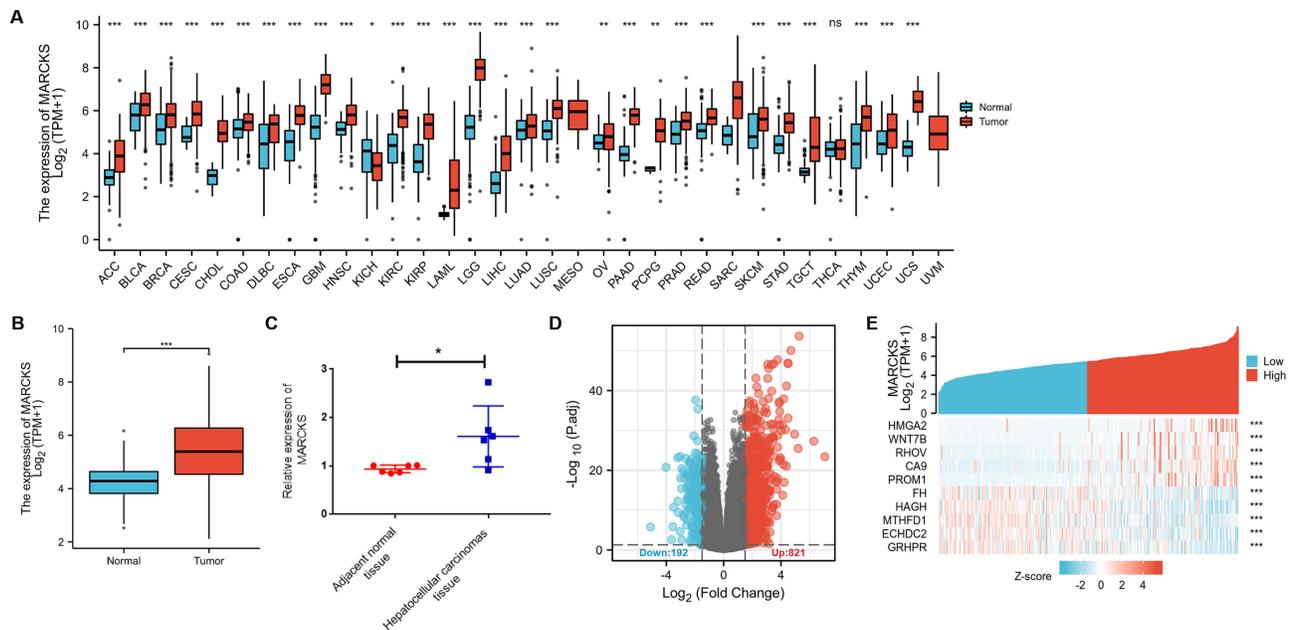
Figure 1 Differential mRNA expression profiles in hepatocellular carcinomas (HCCs) patients stratified by *MARCKS* levels. (**A**) The expression of *MARCKS* in different tumor types and pericancerous tissues was compared based on TCGA database. (**B**) The expression of *MARCKS* in hepatocellular carcinoma was higher than that in pericancerous tissues. (**C**) qPCR was used to detect the expression of *MARCKS* in 8 groups of HCCs and adjacent normal tissues. The high- and low- *MARCKS* DEGs expression groups were shown by volcano plots (**D**). The represented DEGs are presented with heatmaps (**E**). ns, P ≥ 0.05; *P < 0.05; **P < 0.01; ***P < 0.001.

signalling pathway (P.adjusted = 0.040, FDR = 0.031), and WNT signalling pathway in HCC patients (P.adjusted = 0.040, FDR = 0.031), *MARCKS*-associated DEGs were also enriched in the extracellular matrix and T cell receptor signalling pathways.

Correlation Between *MARCKS* Expression and Immune Cell Infiltration in Hepatocellular Carcinomas

Association between *MARCKS* and the infiltration of 24 immune cell types in HCC was analyzed. The results are displayed in Figure 3A. Infiltration of Th2 cells (R = 0.474, P < 0.001), Tfh cells (R = 0.473, P < 0.001), T helper cells (R^2 = 0.419, P < 0.001), macrophages (R^2 = 0.412, P < 0.001), Th1 cells (R^2 = 0.371, P < 0.001), T cells (R^2 = 0.331, P < 0.001), IDCs (R^2 = 0.329, P < 0.001), NK CD56+ cells (R^2 = 0.327, P < 0.001), B cells (R^2 = 0.277, P < 0.001), TEM cells (R^2 = 0.261, P < 0.001) and aDCs (R^2 = 0.235, P < 0.001) were positively correlated with *MARCKS* expression. However, Th17 cells (R^2 = −0.292, P < 0.001) were negatively correlated with *MARCKS* expression. Furthermore, we evaluated the infiltration levels of the 12 most relevant immune cells – Th2 cells (Figure 3B), Tfh cells (Figure 3C), T helper cells (Figure 3D), macrophages (Figure 3E), Th1 cells (Figure 3F), T cells (Figure 3G), IDCs (Figure 3H), NK CD56+ cells (Figure 3I), B cells (Figure 3J), TEM cells (Figure 3K), ADCs (Figure 3L) and Th17 cells (Figure 3M) in the two *MARCKS* expression groups with HCC, and the results are consistent with Figure 3A.

Association of *MARCKS* Expression and Clinicopathological Characteristics in Hepatocellular Carcinomas

We explored the clinicopathological factors of HCC patients in the different *MARCKS* expression groups. Compared with the low-*MARCKS* group, the high-*MARCKS* group had a high proportion, high AFP level, and were young, at advanced pathologic stages, advanced histologic grade and more severe T stages. However, there were no significant differences between the two groups in terms of race, sex, clinical N and M stage distribution, or residual tumours (Table 1).

In addition, we analysed *MARCKS* expression in HCC patients with different clinicopathological characteristics. *MARCKS* was highly expressed in patients with body weights less than 70 kg (Figure 4A) and ages less than 60 years
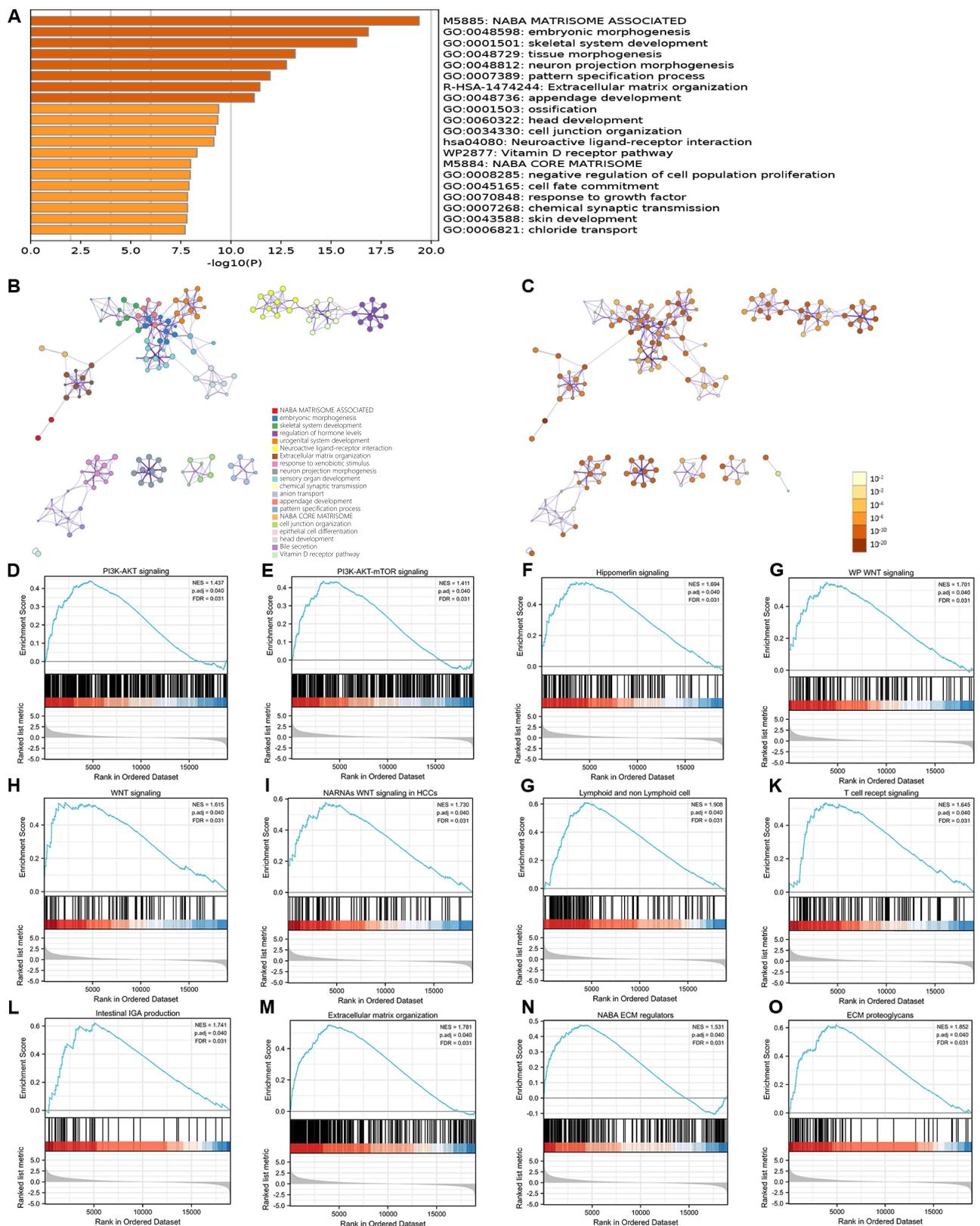
Figure 2 Functional annotation of differentially expressed genes (DEGs) in hepatocellular carcinomas (HCCs) patients with distinct *MARCKS* levels. (**A**) Enrichment pathway of *MARCKS*-related DEGs in Metascape. (**B**) Representative terms were present in a network layout, such as NABA MATRISOME ASSOCIATED, embryonic morphogenesis. (**C**) The same enrichment network presents nodes colored by the *P*-value. (**D**–**O**) Enrichment analysis of representative gene sets of *MARCKS*-related DEGs.
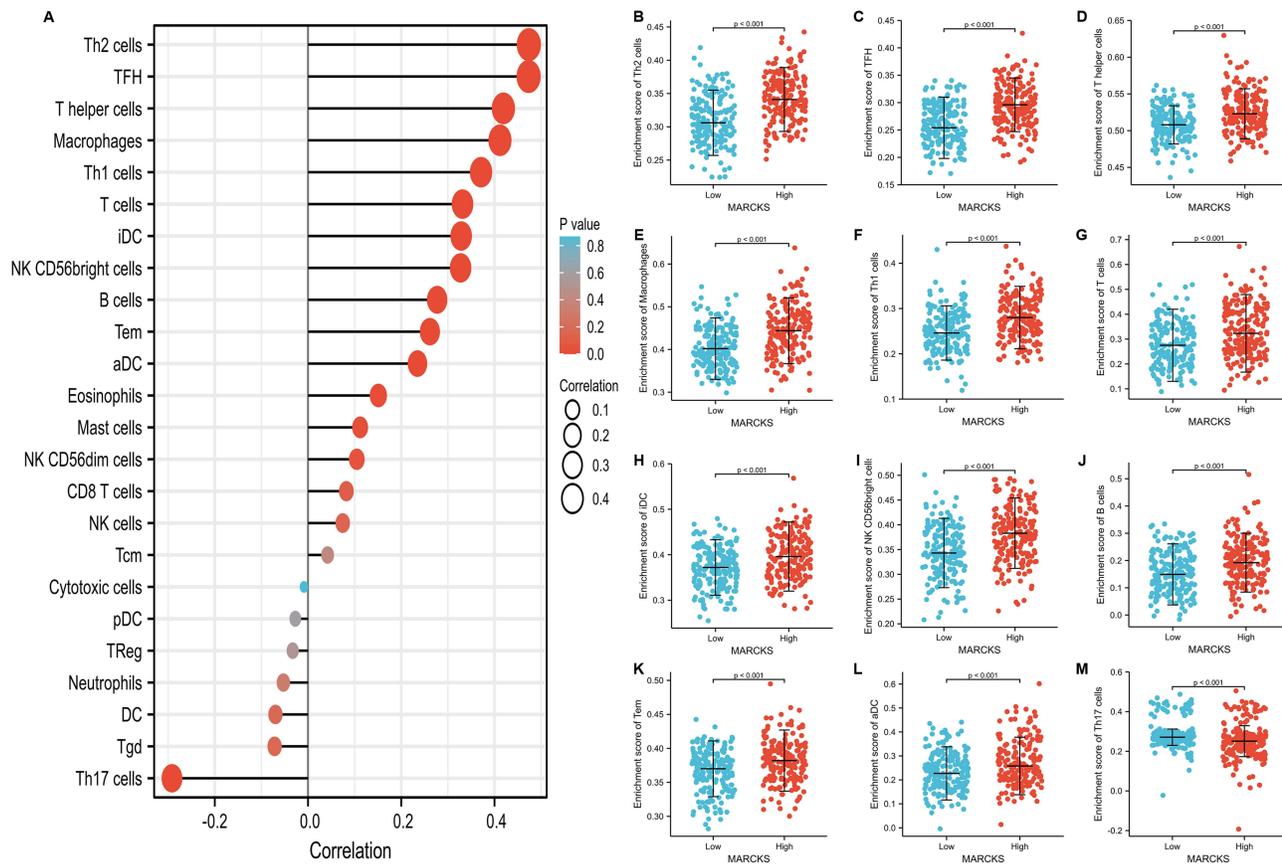
Figure 3 Correlation of immune cell infiltration and *MARCKS* expression in hepatocellular carcinomas (HCC) patients. (**A**) Spearman analysis was used to explore the correlation between the infiltration level of 24 immune cells and the expression of *MARCKS*. Compare the Th2 (**B**), TFH (**C**), T helper (**D**), macrophages (**E**), Th1 (**F**), T (**G**), iDC (**H**), NK CD56+ (**I**), B (**J**), Tem (**K**), aDC (**L**) and Th17 (**M**) cells infiltration levels between the high and low *MARCKS* expression groups.

(Figure 4B), as well as in patients with AFP abnormalities (Figure 4C), tumor status (Figure 4D), and patients at pathological stages III and IV (Figure 4E), histologic stages G3 and G4 (Figure 4F), and T stages T3 and T4 (Figure 4G), and liver fibrosis (Figure 4H). We used Logistic regression model to analyze the correlation between *MARCKS* expression and the clinicopathological characteristics. The results are provided in Table 2. The expression of *MARCKS* was significantly positively correlated with clinical stage (including T stage and tissue stage) and AFP concentration. It was negatively correlated with age and weight.

Table 1 Clinicopathological Characteristics of HCCs Patients with Differential *MARCKS* Expression

Characteristic	Level	Low Expression of *MARCKS* (n=187)	High Expression of *MARCKS* (n=187)
Gender, n (%)			
	Female	56 (15%)	65 (17.4%)
	Male	131 (35%)	122 (32.6%)
Age, n (%)**			
	<=60	75 (20.1%)	102 (27.3%)
	>60	112 (30%)	84 (22.5%)
Race, n (%)			
	Asian	72 (19.9%)	88 (24.3%)
	Black or African American	7 (1.9%)	10 (2.8%)
	White	100 (27.6%)	85 (23.5%)

(*Continued*)

Table 1 (Continued).

Characteristic	Level	Low Expression of *MARCKS* (n=187)	High Expression of *MARCKS* (n=187)
T stage, n (%)*			
	T1	104 (28%)	79 (21.3%)
	T2	45 (12.1%)	50 (13.5%)
	T3	33 (8.9%)	47 (12.7%)
	T4	3 (0.8%)	10 (2.7%)
N stage, n (%)			
	N0	124 (48.1%)	130 (50.4%)
	N1	1 (0.4%)	3 (1.2%)
M stage, n (%)			
	M0	135 (49.6%)	133 (48.9%)
	M1	2 (0.7%)	2 (0.7%)
Pathologic stage, n (%)*			
	Stage I	98 (28%)	75 (21.4%)
	Stage II	43 (12.3%)	44 (12.6%)
	Stage III	31 (8.9%)	54 (15.4%)
	Stage IV	3 (0.9%)	2 (0.6%)
Tumor status, n (%)*			
	Tumor free	112 (31.5%)	90 (25.4%)
	With tumor	67 (18.9%)	86 (24.2%)
Weight, n (%)**			
	<=70	81 (23.4%)	103 (29.8%)
	>70	97 (28%)	65 (18.8%)
Residual tumor, n (%)			
	R0	170 (49.3%)	157 (45.5%)
	R1	5 (1.4%)	12 (3.5%)
	R2	1 (0.3%)	0 (0%)
Histologic grade, n (%)***			
	G1	39 (10.6%)	16 (4.3%)
	G2	98 (26.6%)	80 (21.7%)
	G3	44 (11.9%)	80 (21.7%)
	G4	4 (1.1%)	8 (2.2%)
AFP (ng/mL), n (%)***			
	<=400	137 (48.9%)	78 (27.9%)
	>400	14 (5%)	51 (18.2%)
Age, median (IQR)**		64 (55, 69)	59 (50, 68)

Note: *p<0.05, **p<0.001, ***p<0.0001.
Abbreviation: HCCs, hepatocellular carcinomas.

The Predictive Value of *MARCKS* in the Diagnosis and Prognosis of Hepatocellular Carcinoma

We used the ROC curve to evaluate the value of *MARCKS* expression for the diagnosis of HCC. The results showed that *MARCKS* has high sensitivity and specificity in the diagnosis of HCC (AUC = 0.807, Figure 5A). K-M analysis showed that patients with high *MARCKS* expression had lower survival probabilities (Figure 5B–D). The above analyses prove that *MARCKS* expression has potential as a diagnostic and prognostic indicator of HCC.

The predictive value of *MARCKS* expression for clinical outcomes was evaluated by multivariate COX regression model. The results are shown in Table 3. *MARCKS* was an independent risk factor for overall survival (HR: 2.038, P < 0.01) and disease-specific survival (HR: 2.140, P < 0.05). More importantly, clinical stage, especially T stage, M stage and pathological stage, showed predictive advantages.
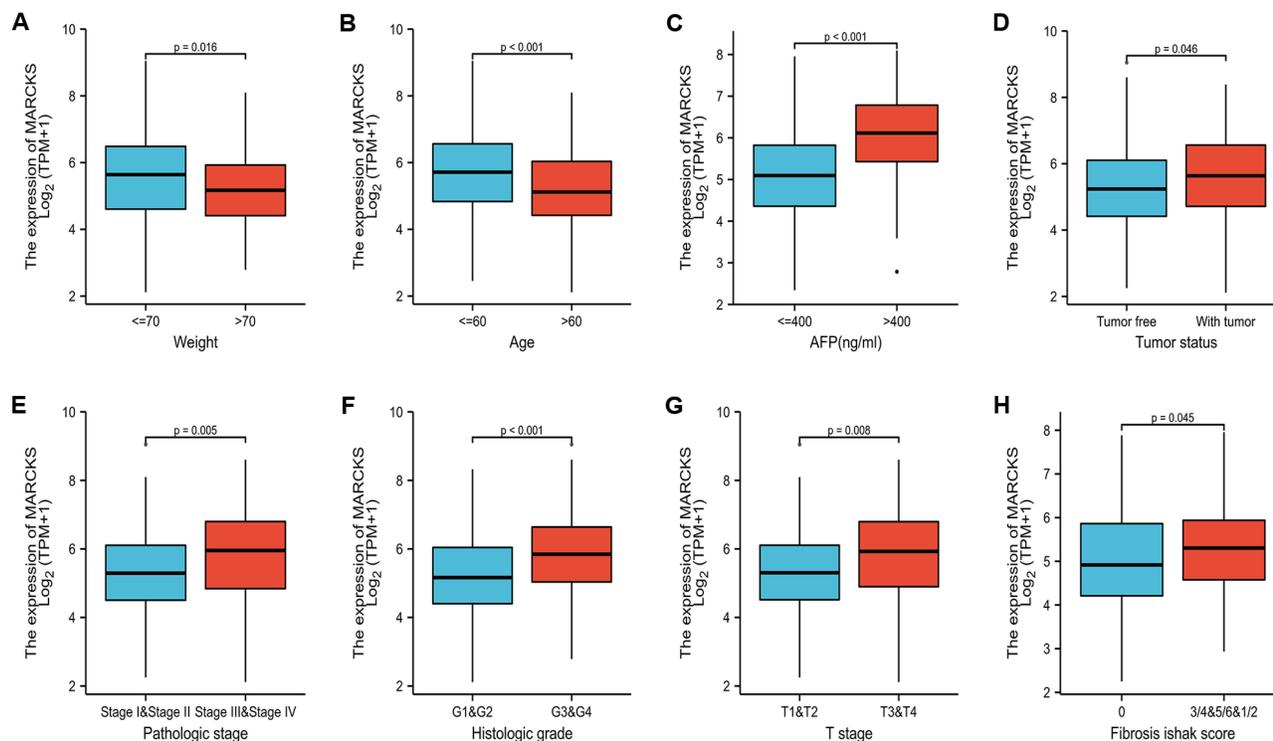
Figure 4 *MARCKS* expression is associated with clinicopathological characteristics in hepatocellular carcinoma (HCCs) patients. The Wilcoxon rank sum test was applied to analyze the association of *MARCKS* expression with weight (**A**), age (**B**), AFP level (**C**), tumor status (**D**), pathologic stage (**E**), Histologic grade (**F**), clinical T stage (**G**), and fibrosis ishak score (**H**).

Next, the statistically significant prognostic factors from the multivariate Cox regression analysis were applied to construct a prognostic nomogram. The calibration curves were drawn to test the validity of the nomogram. Stages of T and M and *MARCKS* expression were included in the overall survival prediction model nomogram with a C-index of 0.663 (Figure 6A). Clinical stages of T, M and *MARCKS* expression were incorporated into the progression-free prediction model of nomogram with a C-index of 0.632 (Figure 6C). Clinical stages T and M and *MARCKS* expression were used to construct a predictive nomogram of disease-specific survival with a C-index of 0.717 (Figure 6E). With the exception of the slightly underestimated prediction of 1-year overall survival, the calibration curves provided good prediction value for all three types of nomograms of clinical outcomes at 1, 3, and 5 years (Figure 6B, D and F).

Prognostic Performance of *MARCKS* in Hepatocellular Carcinomas Clinicopathological Subgroups

Cox regression analysis was performed in the clinicopathological subgroup (Table 4 and Figure 7). *MARCKS* was an important risk factor for overall survival in female patients (HR = 2.82, P = 0.001, Figure 7A), patients at clinical stages

Table 2 Logistic Regression Analysis of Association Between Clinicopathological Characteristics and *MARCKS* Expression in HCCs Patients

| Characteristics	Odds Ratio (OR)	*P* value
T stage (T3&T4&T2 vs T1)	1.739 (1.155–2.629)	0.008
Pathologic stage (IIIV&VI vs I& II)	1.952 (1.200–3.211)	0.008
Age (>60 vs <=60)	0.551 (0.365–0.830)	0.005
Weight (>70 vs <=70)	0.527 (0.342–0.807)	0.003
AFP (ng/mL) (>400 vs <=400)	6.398 (3.410–12.700)	<0.001
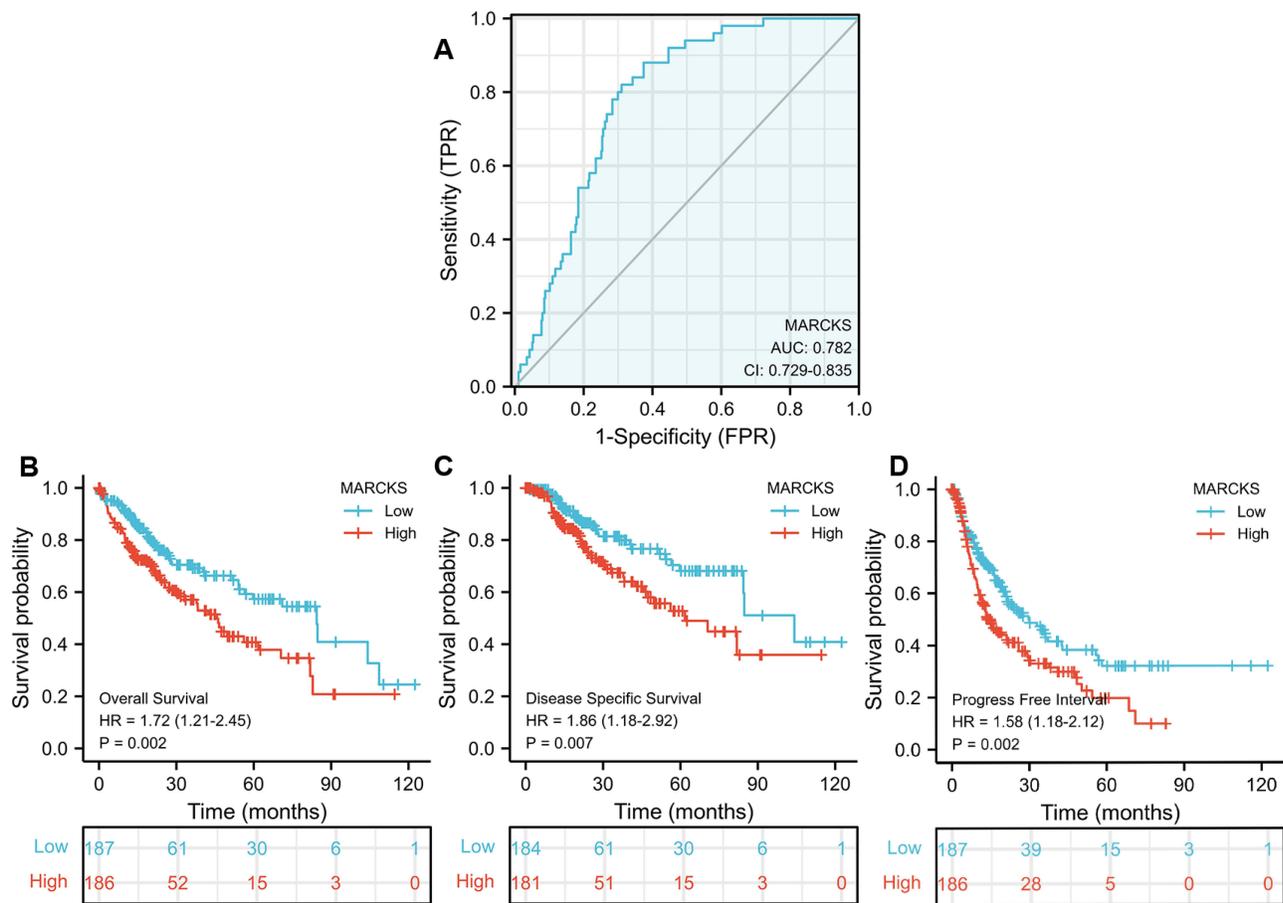
Figure 5 The predictive value of *MARCKS* in the diagnosis and clinical outcome of patients with hepatocellular carcinomas (HCCs). (**A**) ROC curve analysis evaluating the performance of *MARCKS* for HCCs diagnosis. Kaplan–Meier analysis compares the differences in overall survival (OS) (**B**), progression-free survival (PFS) (**C**), and disease-specific survival (DSS) (**D**) between the high and low *MARCKS* expression groups.

III–IV (HR = 2.25, P = 0.019) and clinical T stages T3 and T4 (HR = 2.15, P = 0.008), and patients that weighed less than 70 kg (HR = 2.25, P = 0.003). The same analysis was performed for the progression-free interval (Figure 7B) and disease-specific survival (Figure 7C). Kaplan–Meier analyses were conducted for clinical outcomes in six representative

Table 3 Cox Regression Analysis for Clinical Outcomes in HCCs Patients

Characteristics	HR for Overall Survival (95% CI)		HR for Progression-Free Survival (95% CI)		HR for Disease-Specific survival (95% CI)	
	Univariate	Multivariate	Univariate	Multivariate	Univariate	Multivariate
T stage	2.598 (1.826–3.697)***	2.2 (0.298–16.252)	2.177 (1.590–2.980)***	1.277 (0.306–5.322)	3.639 (2.328–5.688)***	12.275 (0.717–210.177)
N stage	2.029 (0.497–8.281)		1.37 (0.338–5.552)		3.612 (0.870–14.991)	6.279 (0.780–50.549)
M stage	4.077 (1.281–12.973)*	1.799 (0.551–5.879)	3.476 (1.091–11.076)*	2.046 (0.628–6.667)	5.166 (1.246–21.430)*	1.662 (0.385–7.175)
Gender (female vs male)	0.793 (0.557–1.130)		0.982 (0.721–1.338)		0.813 (0.516–1.281)	
Age (<=60 vs >60)	1.205 (0.850–1.708)		0.96 (0.718–1.284)		0.846 (0.543–1.317)	
Weight (<=70 vs >70)	0.941 (0.657–1.346)		1.016 (0.750–1.375)		1 (0.630–1.589)	
Pathologic stage	2.504 (1.727–3.631)***	1.336 (0.182–9.800)	2.201 (1.591–3.046)***	1.659 (0.401–6.861)	3.803 (2.342–6.176)***	0.381 (0.021–6.859)

(Continued)

Table 3 (Continued).

Characteristics	HR for Overall Survival (95% CI)		HR for Progression-Free Survival (95% CI)		HR for Disease-Specific survival (95% CI)	
	Univariate	Multivariate	Univariate	Multivariate	Univariate	Multivariate
AFP (ng/mL)(<=400 vs >400)	1.075 (0.658–1.759)		1.045 (0.698–1.563)		0.867 (0.450–1.668)	
Residual tumor (R0 vs R1&R2)	1.604 (0.812–3.169)		1.513 (0.840–2.726)		1.678 (0.728–3.870)	
MARCKS L (Low vs High)	1.916 (1.345–2.730)***	2.038 (1.303–3.187)**	1.3 (0.972–1.739)	1.218 (0.857–1.732)	1.805 (1.151–2.831)*	2.140 (1.169–3.917)*

Note: *P < 0.05; **P < 0.01; ***P < 0.001.
Abbreviations: HR, hazard ratio; CI, confidence interval.

subgroups: females, ages, patients at clinical stages III–IV, patients at T stages T3 and T4, patients at N stage N0, and patients at M stage M0 (Figure 8). The above results show that the clinical performance of patients with high MARCKS expression is significantly worse.

Discussion

We performed bioinformatics analyses that integrated HCC patient data in TCGA and grouping them based on *MARCKS* expression levels. According to the analysis of *MARCKS* expression and clinical correlations, *MARCKS* expression was significantly different in HCC and adjacent tissues, and the expression of *MARCKS* was significantly related to the sex, age and clinical stage of HCC patients. We also prove that *MARCKS* has potential as a prognostic and diagnostic indicator for HCC patients.

MARCKS is critical in a variety of solid cancers.[35] In our study, we confirmed the expression of *MARCKS* in HCC was upregulated. High expression of *MARCKS* showed clinical significance and was associated with poor survival in HCC patients. These results suggested that *MARCKS* is a potential therapeutic target in HCC. Functional enrichment of *MARCKS* was directly related to many cellular biology processes, including cytoskeletal reorganization, cellular secretion, inflammatory responses, etc.[22] Recent studies reveal that *MARCKS* is closely related to autophagy.[36] In our functional annotation of *MARCKS*-associated DEGs, we found that extracellular matrix organization and cell junction organization were closely associated with *MARCKS* expression. However, more studies are needed to fully understand the mechanisms underlying *MARCKS* overexpression in HCC. Previous studies have shown that inhibition of *MARCKS* inactivates the P3K/AKT pathway,[37] WNT5A can induce the activation of *MARCKS*,[38] and phospho-*MARCKS* is a novel NF-kB activator.[18] GSEA found that *MARCKS* is closely related to the Hippo-Merlin pathway, which has not been confirmed. Hippo-Merlin has been shown to be associated with poor prognosis in HCC,[39] and our results confirmed that high expression of *MARCKS* leads to worse prognosis in HCC patients. The mechanism of *MARCKS* and Hippo-Merlin in poor prognosis of HCC requires further research.

The infiltration of immune cells is closely related to tumour development and prognosis.[40] Our results found that *MARCKS* expression is positively correlated with the infiltration of Th2 and Tfh cells. Increasing evidence supports the association of Th2 cells with the tumour microenvironment, and Th2 cells have been proven to promote tumour growth.[41–43] We hypothesize that *MARCKS* expression may mediate the development of liver cancer by promoting the Th2-type inflammatory response. However, *MARCKS* expression was negatively correlated with Th17 cells. The relative contribution of Th17 cells to tumorigenesis is usually associated with chronic inflammation. Although current evidence regarding the role of Th17 cells in cancer is conflicting, excessive inflammation of Th17 cells may lead to cancer. Therefore, the ability of *MARCKS* to regulate the tumour immune microenvironment requires further research.

At present, the classic markers for the diagnosis of HCC include AFP, AFP-L3, and DCP. AFP has low sensitivity (41–65%) and specificity (80–94%). The diagnostic specificity of DCP is better than that of AFP and AFP-L3, but it
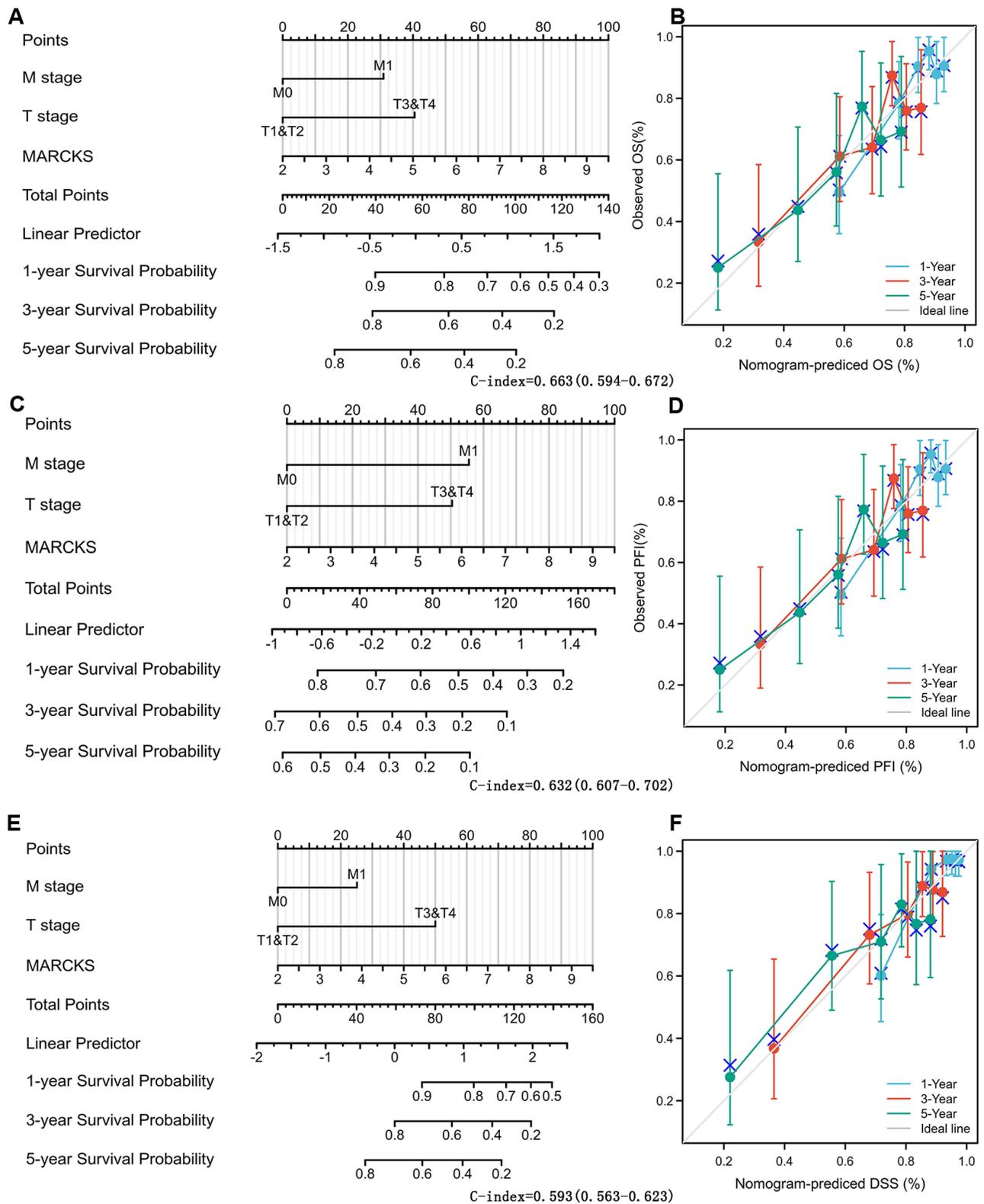
Figure 6 Construction and validation of nomograms based on *MARCKS* expression. A nomogram constructed based on the risk score model of *MARCKS* expression was established for 1, 3, and 5 years overall survival (**A**), progression-free survival (**C**), and disease-specific survival (**E**). The calibration chart verifies the efficiency of the nomogram for overall survival (**B**), progression-free survival (**D**), and disease-specific survival (**F**).

Table 4 Prognostic Performance of *MARCKS* on Clinical Outcomes (OS, PFS and DSS) in HCCs Patient Subgroups by Cox Regression Analysis

Characteristics	N (%)	HR for Overall Survival (95% CI)	HR for Progression-Free Survival (95% CI)	HR for Disease-Specific Survival (95% CI)
Gender				
Female	121 (32.4%)	2.82(1.53–5.19)***	2.46(1.45–4.20)***	3.51(1.58–7.78)**
Male	253 (67.6%)	1.34(1.53–5.19)	1.1(0.77–1.57)	1.4(0.79–2.47)
Age				
<=60	177 (47.5%)	1.83(1.07–3.13)*	1.97(1.28–3.02)**	2.23(1.17–4.24)*
>60	196 (52.5%)	1.71(1.07–2.74)*	1.08(0.72–1.62)	1.68(0.88–3.21)
Pathologic stage				
Stage I & Stage II	260(74.3%)	1.14(0.71–1.85)	1.18(0.82–1.72)	1.06(0.53–2.10)
Stage III & Stage IV	90(25.7%)	2.25(1.24–4.08)**	1.26(0.74–2.13)	2.45(1.18–5.05)*
Clinical T stage				
T1&T2	278(74.9%)	1.29(0.82–2.03)	1.28(0.90–1.83)	1.36(0.73–2.54)
T3&T4	93(25.1%)	2.15(1.22–3.77)**	1.37(0.82–2.29)	2.12(1.08–4.17)*
Clinical N stage				
N0	254 (98.4%)	1.87(1.20–2.92)**	1.71(1.20–2.45)**	2.29(1.27–4.12)**
N0&N1	4 (1.6%)	1.96(1.26–3.06)**	1.7(1.19–2.42)**	2.46(1.37–4.40)**
Clinical M stage				
M0	268 (98.5%)	1.64(1.06–2.54)*	1.65(1.16–2.35)**	1.89(1.07–3.34)*
Weight				
<=70	184 (53.2%)	2.25(1.32–3.84)**	1.46(0.95–2.25)	2.42(1.20–4.87)*
>70	162 (46.8%)	1.58(0.93–2.68)	1.49(0.96–2.29)	2.02(1.01–4.05)*
Residual				
R0	327 (94.8%)	1.53(1.04–2.23)*	1.43(1.05–1.95)*	1.76(1.05–1.95)*
R1&R2	18(5.2%)	0.16(0.02–1.00)*	0.6(0.18–1.98)	0.26(0.03–2.31)
Histologic				
G1&G2	233(63.1%)	1.58(1.00–2.48)*	1.28(0.88–1.87)	1.52(0.85–2.71)
G3&G4	136(36.9%)	1.99(1.11–3.57)*	1.65(1.03–2.65)*	2.19(1.03–4.65)*
AFP (ng/mL)				
<=400	215 (76.8%)	1.23(0.74–2.05)	1.01(0.69–1.48)	1.13(0.60–2.14)
>400	65 (23.2%)	2.01(0.83–4.89)	2.34(1.10–4.97)*	2.76(0.82–9.30)

Note: *P < 0.05; **P < 0.01; ***P < 0.001.
Abbreviations: HR, hazard ratio; CI, confidence interval; OS, overall survival, PFS, progression-free survival, DSS, disease-specific survival (95% CI).

cannot be used for early HCC.[44] Currently, there is a lack of HCC prognostic and diagnostic markers. Our results indicate that *MARCKS* expression can be used as a prognostic and diagnostic marker for HCC. The ROC curve for *MARCKS* expression discrimination of HCC diagnosis had an AUC of 0.782, strongly suggesting that *MARCKS* expression is a convincing biomarker for HCC diagnosis. High expression of *MARCKS* was associated with poor survival. In addition, we demonstrated that *MARCKS* expression is higher in female patients, younger patients, and patients with advanced histological stages, and worse clinical stage pathological features. Moreover, our results indicated that *MARCKS* having greater prognostic value in female patients, patients at clinical stages III–IV and clinical T stages T3 and T4, and patients that weighed less than 70 kg. Therefore, the upregulation and predictive performance of *MARCKS* expression suggests that it may represent a prognostic biomarker for HCC.

We revealed a potential mechanism of *MARCKS* on HCC tumorigenesis and illustrated its predictive value in HCC clinical outcomes, and our study still had several limitations. Further in vivo and in vitro experiments concentrating on mechanism of how *MARCKS* influences the HCC are needed.

A

Characteristics	N(%)	HR(95% CI)	P value
Gender			
Female	121 (32.4%)	2.82(1.53-5.19)	0.001
Male	253 (67.6%)	1.34(1.53-5.19)	0.196
Age			
<=60	177 (47.5%)	1.83(1.07-3.13)	0.028
>60	196 (52.5%)	1.71(1.07-2.74)	0.025
Clinical stage			
Stage I & Stage II	260(74.3%)	1.14(0.71-1.85)	0.58
Stage III & Stage IV	90(25.7%)	2.25(1.24-4.08)	0.008
Clinical T stage			
T1&T2	278(74.9%)	1.29(0.82-2.03)	0.274
T3&T4	93(25.1%)	2.15(1.22-3.77)	0.008
Clinical N stage			
N0	254 (98.4%)	1.87(1.20-2.92)	0.006
N0&N1	4 (1.6%)	1.96(1.26-3.06)	0.003
Clinical M stage			
M0	268 (98.5%)	1.64(1.06-2.54)	0.026
Weight			
<=70	184 (53.2%)	2.25(1.32-3.84)	0.003
>70	162 (46.8%)	1.58(0.93-2.68)	0.089
Residual			
R0	327 (94.8%)	1.53(1.04-2.23)	0.029
R1&R2	18(5.2%)	0.16(0.02-1.00)	0.05
Histologic			
G1&G2	233(63.1%)	1.58(1.00-2.48)	0.049
G3&G4	136(36.9%)	1.99(1.11-3.57)	0.021
AFP(ng/ml)			
<=400	215 (76.8%)	1.23(0.74-2.05)	0.419
>400	65 (23.2%)	2.01(0.83-4.89)	0.123

0 1 2 3 4 5

B

Characteristics	N(%)	HR(95% CI)	P value
Gender			
Female	121 (32.4%)	2.46(1.45-4.20)	0.001
Male	253 (67.6%)	1.1(0.77-1.57)	0.591
Age			
<=60	177 (47.5%)	1.97(1.28-3.02)	0.002
>60	196 (52.5%)	1.08(0.72-1.62)	0.719
Clinical stage			
Stage I & Stage II	260(74.3%)	1.18(0.82-1.72)	0.374
Stage III & Stage IV	90(25.7%)	1.26(0.74-2.13)	0.394
Clinical T stage			
T1&T2	278(74.9%)	1.28(0.90-1.83)	0.174
T3&T4	93(25.1%)	1.37(0.82-2.29)	0.233
Clinical N stage			
N0	254 (98.4%)	1.71(1.20-2.45)	0.003
N0&N1	4 (1.6%)	1.7(1.19-2.42)	0.003
Clinical M stage			
M0	268 (98.5%)	1.65(1.16-2.35)	0.005
Weight			
<=70	184 (53.2%)	1.46(0.95-2.25)	0.085
>70	162 (46.8%)	1.49(0.96-2.29)	0.072
Residual			
R0	327 (94.8%)	1.43(1.05-1.95)	0.023
R1&R2	18(5.2%)	0.6(0.18-1.98)	0.401
Histologic			
G1&G2	233(63.1%)	1.28(0.88-1.87)	0.193
G3&G4	136(36.9%)	1.65(1.03-2.65)	0.039
AFP(ng/ml)			
<=400	215 (76.8%)	1.01(0.69-1.48)	0.972
>400	65 (23.2%)	2.34(1.10-4.97)	0.027

0 1 2 3 4 5

C

Characteristics	N(%)	HR(95% CI)	P value
Gender			
Female	121 (32.4%)	3.51(1.58-7.78)	0.002
Male	253 (67.6%)	1.4(0.79-2.47)	0.79-2.47
Age			
<=60	177 (47.5%)	2.23(1.17-4.24)	0.015
>60	196 (52.5%)	1.68(0.88-3.21)	0.117
Clinical stage			
Stage I & Stage II	260(74.3%)	1.06(0.53-2.10)	0.869
Stage III & Stage IV	90(25.7%)	2.45(1.18-5.05)	0.016
Clinical T stage			
T1&T2	278(74.9%)	1.36(0.73-2.54)	0.331
T3&T4	93(25.1%)	2.12(1.08-4.17)	0.029
Clinical N stage			
N0	254 (98.4%)	2.29(1.27-4.12)	0.006
N0&N1	4 (1.6%)	2.46(1.37-4.40)	0.002
Clinical M stage			
M0	268 (98.5%)	1.89(1.07-3.34)	0.029
Weight			
<=70	184 (53.2%)	2.42(1.20-4.87)	0.014
>70	162 (46.8%)	2.02(1.01-4.05)	0.047
Residual			
R0	327 (94.8%)	1.76(1.05-1.95)	0.021
R1&R2	18(5.2%)	0.26(0.03-2.31)	0.228
Histologic			
G1&G2	233(63.1%)	1.52(0.85-2.71)	0.16
G3&G4	136(36.9%)	2.19(1.03-4.65)	0.042
AFP(ng/ml)			
<=400	215 (76.8%)	1.13(0.60-2.14)	0.696
>400	65 (23.2%)	2.76(0.82-9.30)	0.101

0.0 2.5 5.0 7.5

Figure 7 Prognostic performance of *MARCKS* on clinical outcomes in different HCCs patient subgroups. According to the clinicopathological characteristics of HCC patients, they are divided into different subgroups. Cox regression was used to evaluate the prognostic performance of *MARCKS* for each subgroup overall survival (**A**), progression-free survival (**B**) and disease-specific survival (**C**), and the results were expressed as a hazard ratio. The bar represents the 95% confidence interval of the hazard ratio, and the size of the diamond represents the significance of *MARCKS* performance.
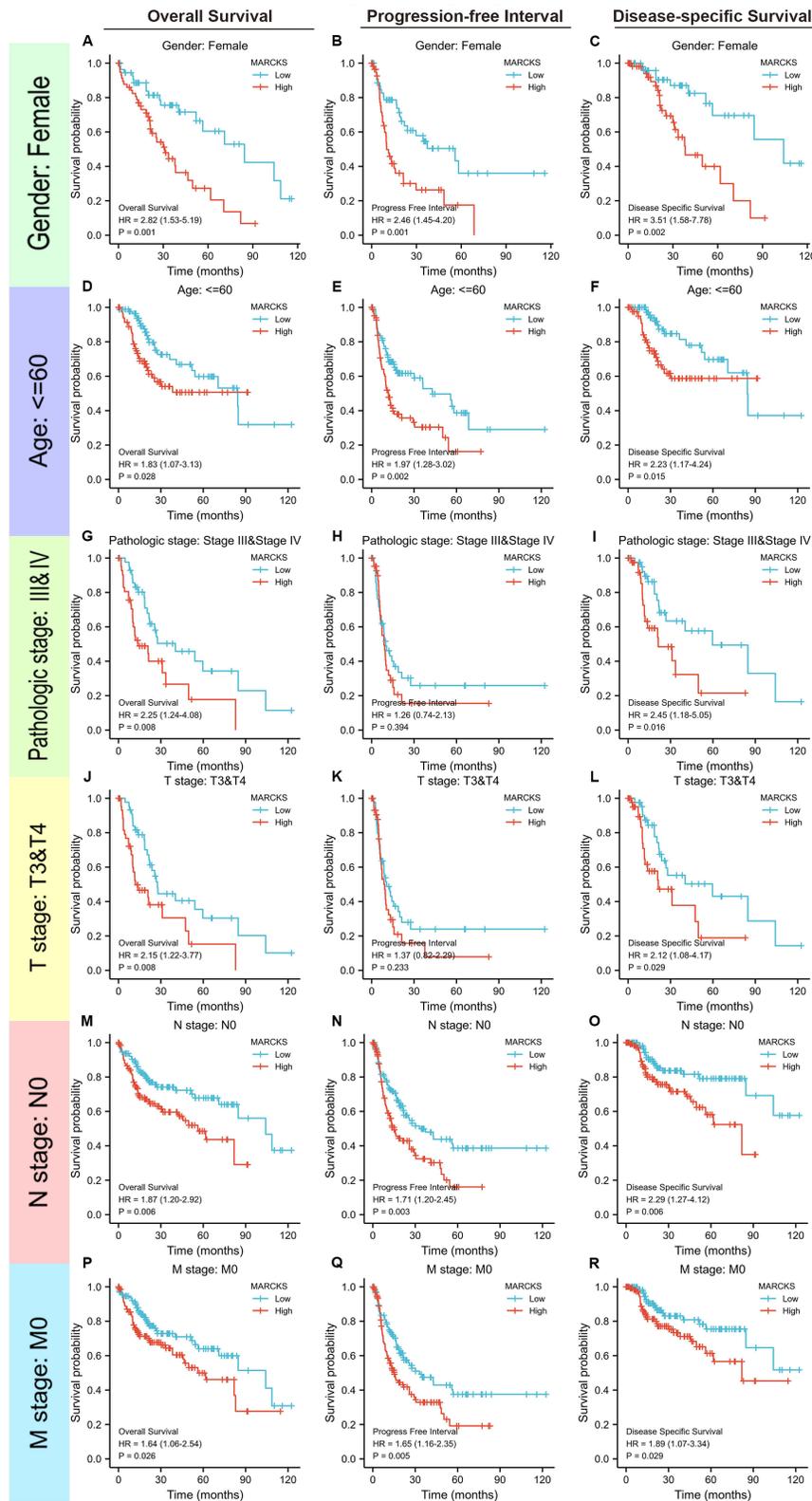
Figure 8 Distinct clinical outcomes based on MARCKS expression in hepatocellular carcinomas (HCCs) patient subgroups. Kaplan–Meier analysis showing the comparison of overall survival (**A, D, G, J, M** and **P**), progression-free survival (**B, E, H, K, N** and **Q**), and disease-specific survival (**C, F, I, L, O** and **R**) between high- and low-MARCKS expression groups in several HCCs patient subgroups, including male gender (**A–C**), age below 60 years (**D–F**), pathologic stage II–IV (**G–I**), T stages T1–T2 (**J–L**), N stage (**M–O**) and M stage (**P–R**).

Conclusion

Increased *MARCKS* expression in HCC might play a role in tumor development and exhibit prognostic value for clinical outcomes.

Abbreviations

AUC, area under the curve; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; ROC, receiver operating characteristic curve; RT-PCR, reverse transcription-polymerase chain reaction.

Data Sharing Statement

The data that support the findings of this study are available in TCGA dataset (https://portal.gdc.cancer.gov/). RNAseq data in level 3 HTSeq-FPKM format of LIHC (hepatocellular carcinoma) project were download from TCGA. To facilitate the use of the data we saved the file to the following website (https://pan.baidu.com/s/13519usqHRrEF3vdPPB24CQ?pwd=yj25). The process of data was described detailedly in manuscript.

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Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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