

Low expression of plakoglobin in intratumoral liver tissue is associated with poor survival after curative resection of Hepatocellular Carcinoma

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Research

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Abstract

Background: Plakoglobin also known as γ -catenin, is a member of the Armadillo family. It's a structural and functional homologue of protein β -catenin with dual functions in cell-cell adhesion and cell signaling. However, the role of plakoglobin in hepatocellular carcinoma (HCC) is unclear to date. Our aim was to investigate the expression of plakoglobin in HCC and its correlation with prognosis and then to further study the underlying function.

Methods: Western Blot and qRT-PCR were used to examine the expression of plakoglobin in HCC cell lines and normal hepatocyte cell line. Immunohistochemical method was used to analyze the expression and relationship of plakoglobin in tissue microarray with 191 HCC patients through Kaplan-Meier analysis.

Results: Patients with high expression of plakoglobin had a better survival compared with those with low expression (OS, $P=0.002$; DFS, $P=0.012$). Plakoglobin is an independent prognostic factor for patients (OS, $P=0.026$, DFS, $P=0.045$). Plakoglobin was inversely consistent with the metastatic potential ability of HCC cell lines.

Conclusions: Our founding indicates plakoglobin is an independent prognostic marker of HCC, and patients with low expression of that had a poor survival. Plakoglobin may serve as a potential target for anti-HCC.

Introduction

Plakoglobin (also known as γ -catenin), discovered more than 30 years ago as an adhesion protein associated with the cell membrane, is a member of the Armadillo family of proteins[1]. Plakoglobin is a structural and functional homologue of protein β -catenin, containing a central armadillo repeat domain[2]with dual functions in cell-cell adhesion and cell signaling[3, 4]. Both plakoglobin and β -catenin could interact with the cytoplasmic domain of cadherins, such as E-cadherin, tethering the cadherin-catenin complex to the cytoskeleton, though plakoglobin was found to be only loosely associated with E-cadherin[3]. In addition, several evidences suggest that plakoglobin has signaling ability similar to β -catenin when it's not sequestered at the plasma membrane as a part of the desmosomes, plakoglobin is capable to participate in cell signaling[5, 6]. However, unlike β -catenin that has a well-defined oncogenic potential as the terminal component involved in the Wnt signaling pathway[7, 8], plakoglobin is generally considered as a tumor or metastasis suppressor[9, 10], although its role in cancer is still controversial[3]. Recent some studies suggest that plakoglobin acts as a tumor-suppressor protein in some tumors, such as lung primary cancer, prostate cancer and esophageal cancer, its expression in these cancers is very low or completely absent[11, 12]. Whereas other studies suggest that plakoglobin has a tumor-inducing effect or oncogenic activity[13–15]. It is possible that plakoglobin may have different roles in different malignancies. Recently, an immunohistochemical study revealed that 58% of human hepatocellular carcinoma (HCC) cases showed that reduced levels of E-cadherin associated with a declined expression of p120 catenin, α - and β -catenin and plakoglobin in a comparable percentage of HCC patients[16].

Additionally, our previous study revealed that β -catenin could be activated by hypoxia in HCC and contributed to enhance the metastatic potential and poor prognosis[17]. In this situation, to investigate exact role of plakoglobin involved in HCC is an outstanding issue.

HCC is one of the most common type of primary tumors in the liver with increasing incidence. It is the second most common cause of cancer death in men and sixth-greatest cause of cancer death in women worldwide[18]. Only about 20%-30% of HCC patients are curable by surgical resection with a reported 5-year 40%-50% survival rate and a high incidence of postoperative recurrence rate[19, 20], most patients diagnosed HCC are not candidate for curative treatments[21]. Postoperative recurrence may occur as a result of intrahepatic metastasis or de novo tumor occurrence[22]. Therefore, understanding the mechanism of tumor recurrence after curative resection is crucial for relieving this global health burden and improving the outcome of HCC patients. Metastasis and tumorigenesis is a multi-step process that the tumor cells acquire the ability to degrade the basement membrane and move from the primary site to distant sites forming the secondary tumors, which is the major cause of recurrence and cancer-related deaths. During this process, the intercellular adhesive complexes (mainly called adherens junctions and desmosomes) do limit the abnormal proliferation, migration and invasion and maintain the tissue integrity[23, 24]. However, loss of the desmosomal adhesion is a precondition for the epithelial-mesenchymal transition, indicated that the conversion of early stage primary tumors to invasive cancers.

In this study, to investigate the significance of plakoglobin in HCC patients' prognosis, we detected the expression of plakoglobin and analyzed the correlation between its expression and clinic pathological parameters in HCC patients. Moreover, we evaluated the expression of plakoglobin in several human HCC cell lines. Our results support the hypothesis that plakoglobin may act as a tumor suppressor in HCC.

Materials And Method

Patients and specimens

In our study, a total of 191 HCC specimens were collected randomly from patients who underwent radical resection of pathologically confirmed tumors from 1999 to 2006 at the Liver Cancer Institute and Zhongshan Hospital (Fudan University, Shanghai, China). No patients received any preoperative anticancer treatment. The ethics committee of Zhongshan Hospital approved this research. All patients provided written informed consent to participate in this study.

Tissue microarray (TMA) and Immunohistochemistry (IHC)

A tissue microarray (TMA) was constructed as described previously[25]. Briefly, two cores were taken from each representative tumor tissue and from peritumoral liver tissue (in collaboration with Shanghai Biochip Company Ltd, Shanghai, China). Duplicate cylinders from two different areas, intratumoral and peritumoral (a total of four punches for each patient), were obtained. The rabbit anti-plakoglobin (Cat. No. A303-718A, CST) was used in the immunochemistry to investigate the expression. The density of positive staining was measured with the use of a computerized image system composed of a Leica CCD camera

DFC420 connected to a Leica DM IRE2 microscope (Leica Microsystems Imaging Solutions Ltd, Cambridge, United Kingdom).

Cell lines and Culture Conditions

Four HCC cell lines (HepG2, HCCLM3, MHCC97L and MHCC97H) and human hepatic cell line L02 were used in cell experiments. HCCLM3, MHCC97L and MHCC97H are human HCC cell lines with high metastatic potential[26] (established at the liver cancer institute, Zhongshan Hospital, Fudan University, Shanghai, China) and HepG2 is HCC cell line with low metastatic potential (provided by the liver cancer institute, Zhongshan Hospital, Fudan University, Shanghai, China). L02 is normal liver cell line (obtained from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal bovine serum (FBS; Invitrogen GIBCO), 100U ml⁻¹ penicillin and 50 µg ml⁻¹ streptomycin.

RNA Isolation and qRT-PCR

Total RNA was extracted from the four HCC cell lines (Hep G2, MHCC97L, MHCC97H and HCCLM3) and one normal liver cell line (L02) using Trizol reagent (Invitrogen) according to the manufacturer's instructions. qRT-PCR was performed using a ABI SYBR Green qPCR super Mix Kit according to the manufacturer's instructions. GAPDH was used as an internal control. The primers were as follows: plakoglobin forward primer 5'- ATGGAGGTGATGAACCTGATGGAG-3' and reverse primer 5'- TTGAGCGTGTACTGGCGCCC-3' and GAPDH forward primer 5' -TTCACCACCATGGAGAAGG -3' and reverse primer 5' -GGCATGGACTGTGGTCATGA-3'. Relative mRNA levels were calculated based on Ct values, corrected for GAPDH expression, according to the equation: $K \times 2^{-\Delta Ct}$ [$\Delta Ct = Ct(\text{plakoglobin}) - Ct(\text{GAPDH})$], while K is a constant. All the experiments were performed in triplicate.

Western Blot

Cells were lysed using cell lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 1% Triton X-100) containing protease and phosphatase inhibitors. Equivalent amounts of whole cell extracts were subjected to SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk for 2 h and then incubated with respective primary antibody overnight at 4°C, followed by the incubation of the appropriate HRP-conjugated secondary antibody for 2 h at room temperature. Blots were visualized with an ECL detection kit (Pierce, IL) and analyzed using Quantity One 1-D Analysis Software (Bio-Rad, San Francisco, CA).

Statistical analysis

Data analysis was performed with SPSS 20.0 for Windows (SPSS Inc. Chicago, IL, USA). Unpaired Student's t-test or Pearson's χ^2 test was used to compare quantitative variables. Kaplan-Meier analysis

was used to determine the survival and recurrence, the Cox regression model was used to perform multivariate analysis. $P < 0.05$ was considered statistically significant.

Results

1. Patient characteristics and intratumoral plakoglobin expression

We summarized the 191 HCC patient (160 males, 31 females) characteristics in Table 1. In this study, basic clinicopathological features, including age, gender, hepatitis B surface antigen (HBs-Ag), liver cirrhosis, α -fetoprotein, tumor size, tumor encapsulation, microvascular invasion, tumor differentiation and TNM stage were collected (Table 1). According to the expression of plakoglobin in intratumoral tissues, these patients were classified into the high and low group, we investigated the relationship between plakoglobin expression and clinicopathological features. The results revealed that plakoglobin was statistically significant association with age, gender, HBs-Ag ($P = 0.017$), microvascular invasion ($P = 0.014$) and TNM stage ($P = 0.040$ and $P = 0.027$). However, none of the other clinicopathological features such as α -fetoprotein (AFP), liver cirrhosis, tumor size, encapsulation or differentiation was correlated with plakoglobin expression in intratumoral tissues.

2. Low expression in HCC patients showed poor prognosis

We analyzed tissue from 191 patients with HCC using immunohistochemistry (IHC). Plakoglobin staining was mainly on the plasma membrane of tumor cells or hepatocytes (Figure 1A), the results revealed that patients with high expression of plakoglobin had a better overall survival (OS, $P = 0.002$) and a better disease free survival (DFS, $P = 0.012$) (Figure 1B). There were no statistically significant associations between the peritumoral plakoglobin expression and the OS ($P = 0.145$) and DFS ($P = 0.377$) rate after radical surgical resection of HCC (Figure 1C). In addition to plakoglobin expression, univariate analysis revealed that AFP-1 ($P = 0.033$), tumor differentiation ($P = 0.005$), tumor size ($P < 0.001$) and microvascular invasion ($P < 0.001$) were associated with OS rate of patients with HCC. However, only the tumor size ($P = 0.022$) and microvascular invasion ($P = 0.003$) were associated the DFS of HCC patients (Table 2). Multivariate Cox's proportional Hazard model analysis suggested that high expression of plakoglobin as an independent prognostic factor for the OS ($P = 0.026$) and DFS ($P = 0.045$) rates of HCC patients (Table 3). Besides, microvascular invasion was also an independent prognostic factor for HCC. Moreover, we detected the expression of plakoglobin by western blot in four pairs tumor and matched peritumoral tissues randomly collected from four patients (Figure 1D). The results showed that the expression of plakoglobin was lower in tumor tissues than that in peritumoral tissues.

3. Expression level of plakoglobin in HCC cell lines

We detected the expression of plakoglobin mRNA by qPCR in four HCC cell lines (Figure 2A), and found that the expression level of plakoglobin mRNA was higher in L02 cell, and lower in HCC cell lines; moreover, the expression level of plakoglobin was inversely consistent with the metastatic potential ability of HCC cell lines. Furthermore, we detected the expression level of plakoglobin by western blot

analysis in the above-mentioned five cell lines (Figure 2B), which showed that the result was consistent with that detected by qPCR.

Discussion

Plakoglobin was found more than thirty years ago as an adhesion protein associated with the cell membrane, it's a homologue of protein β -catenin. Although the role of β -catenin, an important component of the canonical Wnt signaling pathway, is well understood in various aspects of liver pathology, there is little information on the role of plakoglobin. The most documented role of plakoglobin within the cell is in cell-cell adhesion as a desmosomal protein. Recent emerged evidence has demonstrated that plakoglobin has novel roles in intracellular signaling and the regulation of gene expression. Similar to β -catenin, plakoglobin has two major roles in the cell: the mediation of cell-cell adhesion and cellular signaling, sometimes replace β -catenin for its function in the Wnt signaling pathway[27]. Despite some reports revealed that plakoglobin expression induced the mesenchymal-epithelial transition (MET) and suppressed tumorigenesis and metastasis, the underlying mechanisms that could account for its function activities had remained unclear, though the induction of MET by plakoglobin may occur via a common pathway involved β -catenin[28]. Some studies have suggested a tumor promoting activity of plakoglobin in malignancies; while others suggested it acted as a tumor suppressor, plakoglobin loss correlates with tumor progression, metastasis and poor prognosis. Loss of plakoglobin is thought to enhance the β -catenin signaling due to reduced competition for Lef/Tcf binding[29]. In breast cancer, Holen et al. found that plakoglobin acted as a tumor suppressor, loss of plakoglobin promoted the decreasing of cell-cell contact, and increased the invasive behavior of breast cancer cells in vivo [30]; however, the results of Aceto et al. suggested that plakoglobin acted as a metastasis promoter in breast cancer circulating tumor cells[14]. The role of plakoglobin in solid tumors existed controversy. While the role of plakoglobin in hepatocellular carcinoma had remained unclear.

In our study, we preliminary explored the expression of plakoglobin and its relationship with HCC. Our results revealed that HCC patients with high expression of plakoglobin had a better prognosis than those with low expression, and the expression of plakoglobin in peritumoral tissues showed no significance with the prognosis. To some extent, our results were consistent with some reported studies, suggesting that plakoglobin acted as a suppressor in hepatocellular carcinoma. Although the precise mechanisms of plakoglobin function on hepatocellular carcinoma development need to be further investigated, our study confirms the value of plakoglobin in the prediction of prognosis and the development of new therapeutic interventions.

Conclusions

In conclusion, our founding indicates plakoglobin is an independent prognostic marker of HCC, and patients with low expression of that had a poor survival. Plakoglobin may serve as a potential target for anti-HCC.

Abbreviations

HCC, hepatocellular carcinoma; MET, mesenchymal-epithelial transition; TMA, tissue microarray; HBs-Ag, hepatitis B surface antigen

Declarations

Ethics approval and consent to participate

Zhongshan Hospital Research Ethics Committee approved the present study. Written informed consent for the use of their tissue and clinicopathological data was obtained from all patients.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interest

The authors declare that they have no conflicts of interest.

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Authors' contributions

Author contribution as follows: De-Ning Ma and Zhi-Xuan Fu designed the experiments; De-Ning Ma and Zhuo Liu analyzed the clinicopathological data and performed the survival analysis. Jie Yuan, Xin-Yi Gao and Dian-Dian Sun performed the experiments and collected the follow-up information of patients; and De-Ning Ma wrote the paper. All authors read and approved the final manuscript.

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Tables

Table 1. Intratumoral plakoglobin expression and clinicopathological features

Variables	Intratumoral plakoglobin expression (n=191)		
	Low (n = 91)	High (n =100)	P value
	No. of patients 47.64%	No. of patients 52.36%	
Age, year†	50.24 ± 11.271	54.77 ± 10.846	0.005
Gender			0.040
Male	71	89	
Female	20	11	
HBs-Ag			0.017*
Positive	83	78	
Negative	8	21	
AFP-1			0.521
≤200ng/mL	44	53	
>200 ng/mL	47	47	
AFP-2			0.207
≤20ng/mL	25	36	
>20 ng/mL	66	64	
Liver cirrhosis			0.713
Yes	23	23	
No	68	77	
Tumor size, cm†	5.919 ± 4.127	5.110 ± 3.362	0.138
Tumor encapsulation			0.875*
Complete	44	49	
None	47	50	
Microvascular invasion			0.014
Yes	47	34	
No	44	66	
Tumor differentiation			0.922
I-II	67	73	
III-IV	24	27	
TNM stage-			0.040
I	11	10	
II	30	51	
IIIA	50	39	
TNM stage			0.027
I-II	41	61	
III-IV	50	39	

†Student's t-test. AFP: a-fetoprotein; HBs-Ag: Hepatitis B virus surface antigen; TNM: tumor-node-metastasis.

*Missing data.

Table 2. Univariate analyses of factors associated with survival and recurrence

Factors	OS, P value	DFS, P value
Age: <55 vs ≥55 years	0.849	0.677
Gender: female vs male	0.343	0.588
HBs-Ag	0.393	0.705
Liver cirrhosis: yes vs no	0.303	0.744
AFP-1: >200ng/mL vs ≤200ng/mL	0.033	0.106
AFP-2: >20ng/mL vs ≤20ng/mL	0.104	0.132
Tumor differentiation: III-IV vs I-II	0.005	0.122
Tumor size: >5 cm vs ≤5 cm	0.000	0.022
Tumor encapsulation: complete vs none	0.795	0.870
Microvascular invasion: yes vs no	0.000	0.003
Intratumoral plakoglobin high vs low	0.002	0.012

Abbreviations: AFP: a-fetoprotein; OS, overall survival; DFS, disease-free survival.

Table 3. Multivariate analyses of factors associated with survival and recurrence

Factors	OS				DFS			
	Univariate <i>P</i>	Multivariate			Univariate <i>P</i>	Multivariate		
		Hazard Ratio	95% CI	<i>P</i>		Hazard Ratio	95% CI	<i>P</i>
AFP-1: >200ng/mL vs ≤200ng/mL	0.033			NA	0.106			NA
Tumor differentiation: III-IV vs I-II	0.005			NA	0.122			NA
Tumor size: >5 cm vs ≤5 cm	0.000	0.378	0.221-0.648	0.000	0.022	0.663	0.421-1.044	0.076
Microvascular invasion: yes vs no	0.000	0.342	0.202-0.580	0.000	0.003	0.590	0.373-0.934	0.024
Intratumoral plakoglobin high vs low	0.002	0.577	0.342-0.974	0.026	0.012	1.597	1.011-2.523	0.045

Abbreviation: AFP: a-fetoprotein. NA: not adopted.

Figures

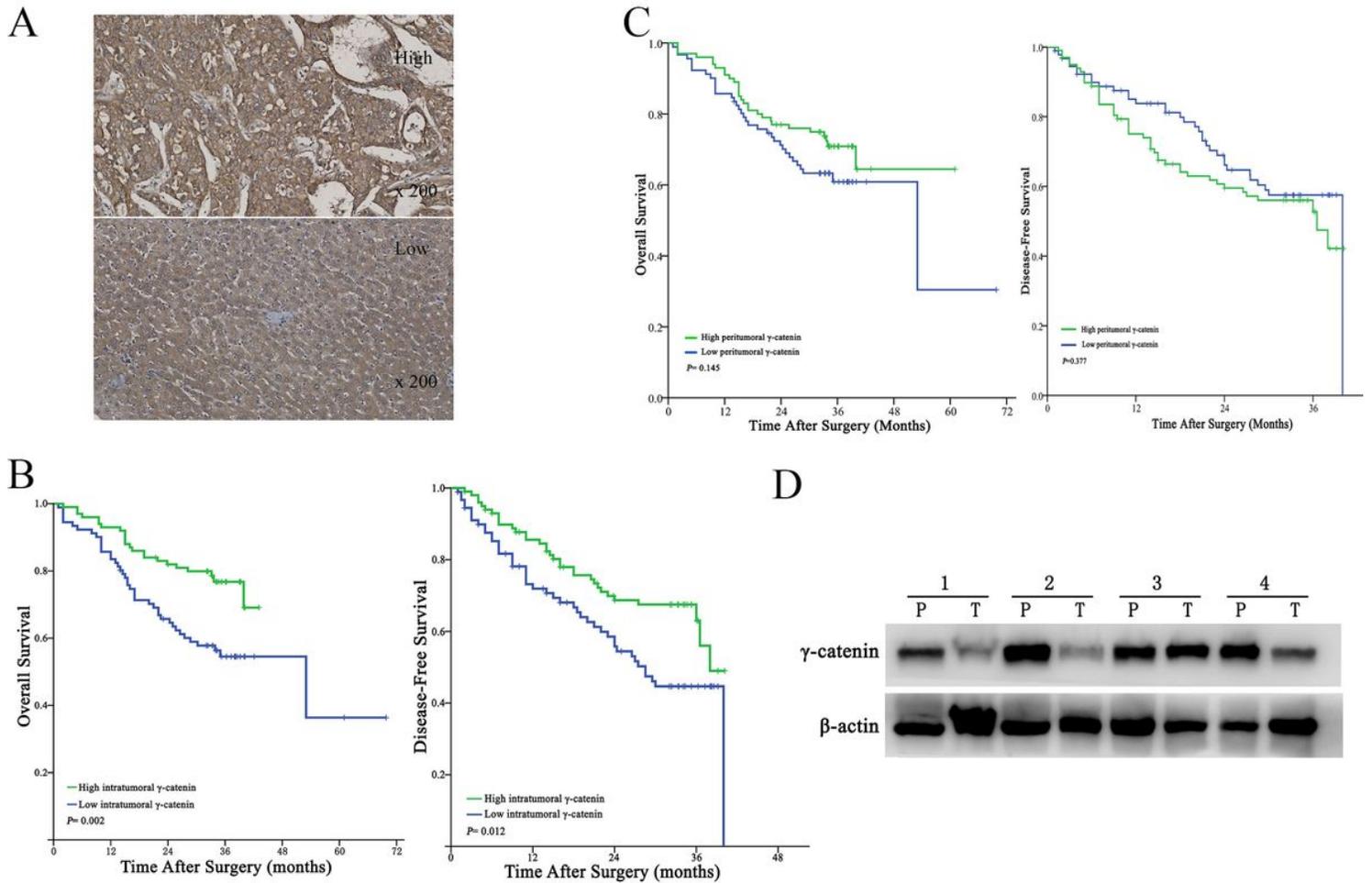


Figure 1

1 A, Immunohistochemistry staining (magnification, X200), demonstrated the expression of plakoglobin in HCC tissues. B, Overall survival and Disease-free survival according to the intratumoral expression of plakoglobin. C, Overall survival and Disease-free survival according to the peritumoral expression of plakoglobin. D, Western blot assay showed the expression of plakoglobin in four paired fresh tissues (tumor tissue and peritumoral tissue)

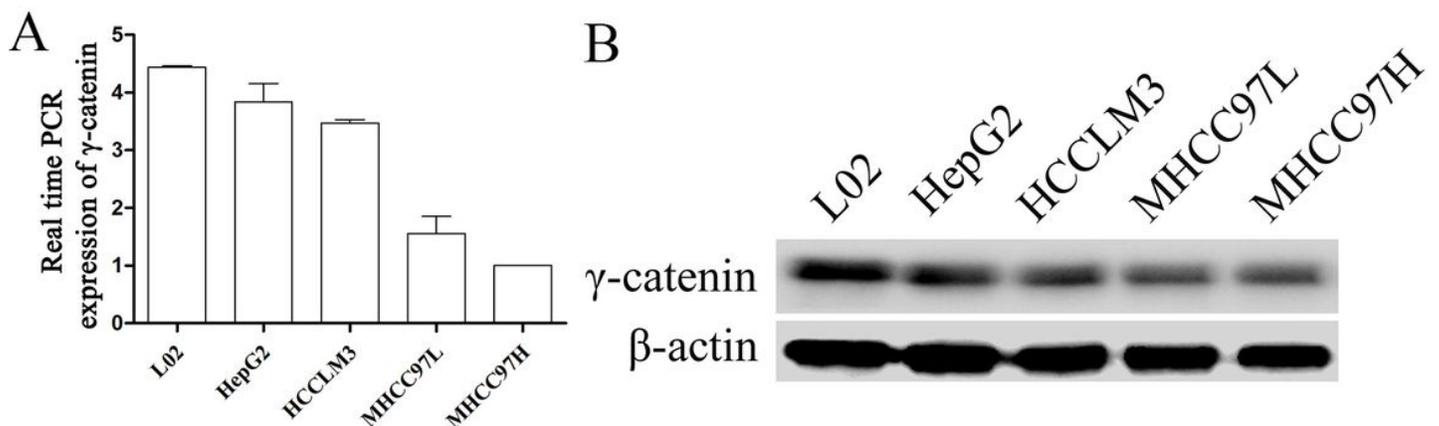


Figure 2

A, qRT-PCR assay showed the expression of plakoglobin in liver cancer cells and normal liver cell. B, Western blot assay verified the expression of plakoglobin in the above-mentioned cells.