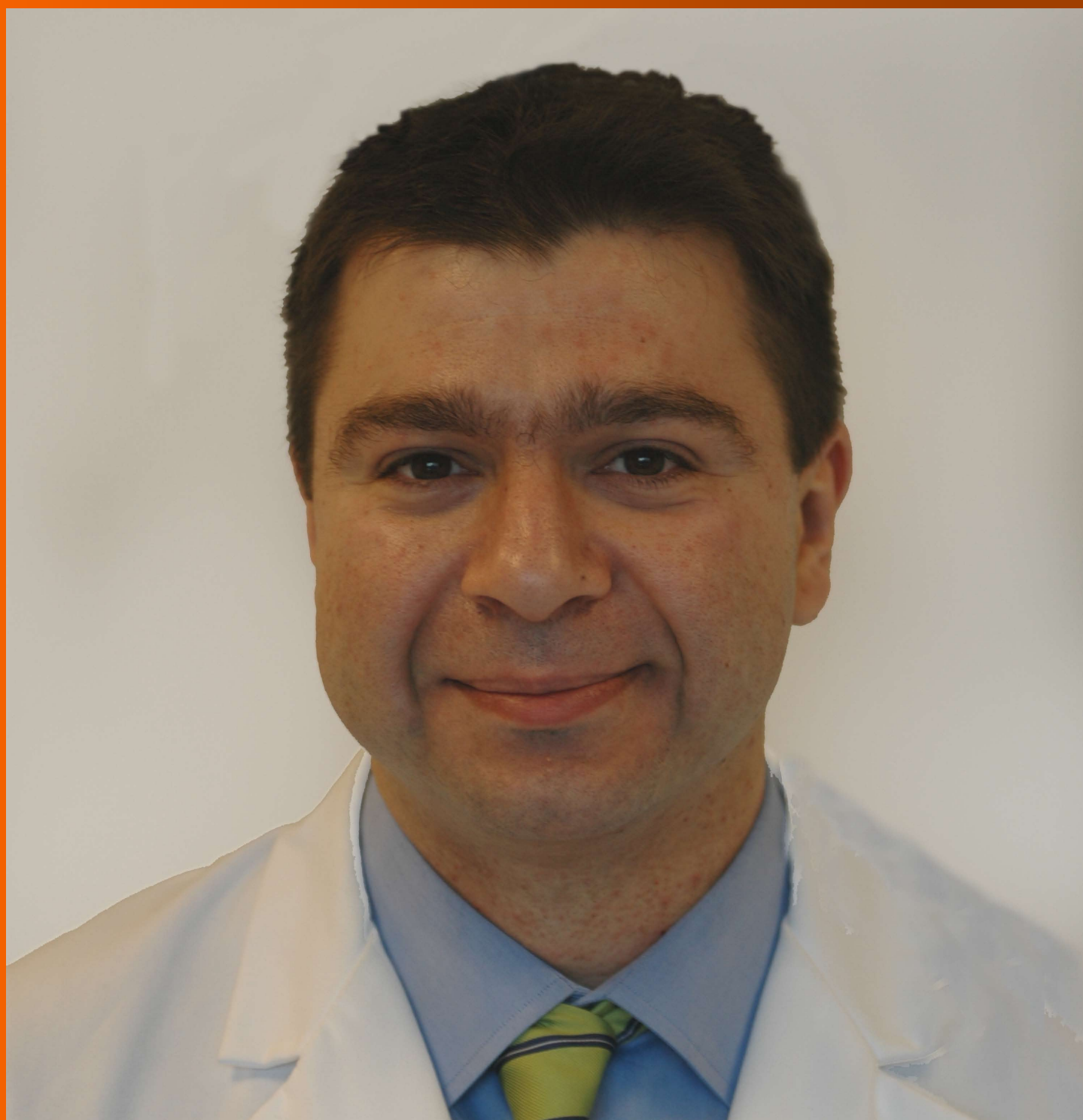


World Journal of *Gastroenterology*

World J Gastroenterol 2018 March 14; 24(10): 1063-1180



Clinical Practice Study

Progesterone receptor membrane component 1 as a potential prognostic biomarker for hepatocellular carcinoma

Hung-Wen Tsai, Chung-Liang Ho, Shu-Wen Cheng, Yih-Jyh Lin, Chou-Cheng Chen, Pin-Nan Cheng, Chia-Jui Yen, Ting-Tsung Chang, Po-Min Chiang, Shih-Huang Chan, Cheng-Hsun Ho, Shu-Hui Chen, Yi-Wen Wang, Nan-Haw Chow, Jou-Chun Lin

Hung-Wen Tsai, Po-Min Chiang, Institute of Clinical Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 70403, Taiwan

Hung-Wen Tsai, Chung-Liang Ho, Shu-Wen Cheng, Yi-Wen Wang, Nan-Haw Chow, Jou-Chun Lin, Department of Pathology, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 70403, Taiwan

Hung-Wen Tsai, Chung-Liang Ho, Shu-Wen Cheng, Chou-Cheng Chen, Nan-Haw Chow, Jou-Chun Lin, Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan 70403, Taiwan

Hung-Wen Tsai, Ting-Tsung Chang, Center of Infectious Disease and Signaling Research, College of Medicine, National Cheng Kung University, Tainan 70403, Taiwan

Yih-Jyh Lin, Department of Surgery, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 70403, Taiwan

Pin-Nan Cheng, Chia-Jui Yen, Ting-Tsung Chang, Cheng-Hsun Ho, Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 70403, Taiwan

Shih-Huang Chan, Department of Statistics, College of Management, National Cheng Kung University, Tainan 70403, Taiwan

Cheng-Hsun Ho, Research Center of Clinical Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan 70403, Taiwan

Shu-Hui Chen, Department of Chemistry, College of Sciences, National Cheng Kung University, Tainan 70403, Taiwan

ORCID number: Hung-Wen Tsai (0000-0001-9223-2535); Chung-Liang Ho (0000-0002-4562-5122); Shu-Wen Cheng

(0000-0002-8684-4428); Yih-Jyh Lin (0000-0003-0998-3229); Chou-Cheng Chen (0000-0002-4030-8849); Pin-Nan Cheng (0000-0001-9331-9018); Chia-Jui Yen (0000-0001-8744-6248); Ting-Tsung Chang (0000-0002-5073-2678); Po-Min Chiang (0000-0002-4950-9292); Shih-Huang Chan (0000-0002-3666-180X); Cheng-Hsun Ho (0000-0003-4451-2887); Shu-Hui Chen (0000-0003-1871-9366); Yi-Wen Wang (0000-0001-7863-727X); Nan-Haw Chow (0000-0002-0277-1947); Jou-Chun Lin (0000-0002-2358-3670).

Author contributions: Lin JC, Cheng SW, Wang YW and Tsai HW conducted the experiments in this study, analyzed the data, and prepared the manuscript; Tsai HW, Ho CL, Chiang PM and Chow NH performed the pathological analysis; Lin YJ, Cheng PN, Yen CJ and Chang TT provided and analyzed the clinical information; Chen CC performed the TCGA data analysis; Chan SH conducted the statistical analysis; Ho CH and Chen SH performed the mass spectrometry and data analysis; Tsai HW designed the study.

Supported by the Ministry of Science and Technology, No. NSC102-2320-B-006-011., No. MOST103-2320-B-006-021-MY2, and No. MOST105-2320-B-006-033 to Tsai HW; and National Cheng Kung University Hospital, Taiwan, No. NCKUH-10406002 and No. NCKUH-10509001 to Tsai HW.

Institutional review board statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Human Experiment and Ethics Committee of NCKUH (No. BR-100-117).

Conflict-of-interest statement: The authors declare that they have no competing interests.

Data sharing statement: Technical appendix and study data are available from the corresponding author at hungwen@mail.ncku.edu.tw with the permission of Hung-Wen Tsai. Consent was not obtained but the presented data are anonymized and the risk of identification is low. No additional data are available.

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Manuscript source: Unsolicited manuscript

Correspondence to: Hung-Wen Tsai, MD, Associate Professor, Department of Pathology, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, 138 Sheng-Li Road, Tainan 70403, Taiwan. hungwen@mail.ncku.edu.tw
Telephone: +886-6-2353535-2635
Fax: +886-6-2766195

Received: December 11, 2017

Peer-review started: December 12, 2017

First decision: December 27, 2017

Revised: January 16, 2018

Accepted: January 24, 2018

Article in press: January 24, 2018

Published online: March 14, 2018

Abstract

AIM

To investigate the clinicopathological significance of progesterone receptor membrane component 1 (PGRMC1) and PGRMC2 in hepatocellular carcinoma (HCC).

METHODS

We performed immunohistochemical staining to evaluate the estrogen receptor (ER), progesterone receptor (PR), PGRMC1, and PGRMC2 in a clinical cohort consisting of 89 paired HCC and non-tumor liver samples. We also analyzed HCC data ($n = 373$) from The Cancer Genome Atlas (TCGA). We correlated the expression status of PGRMC1 and PGRMC2 with clinicopathological indicators and the clinical outcomes of the HCC patients. We knocked down or overexpressed PGRMC1 in HCC cell lines to evaluate its biological significance in HCC cell proliferation, differentiation, migration, and invasion.

RESULTS

We found that few HCC cases expressed ER (5.6%) and PR (4.5%). In contrast, most HCC cases expressed PGRMC1 (89.9%) and PGRMC2 (100%). PGRMC1 and PGRMC2 exhibited significantly lower expression in tumor tissue than in non-tumor tissue ($P < 0.001$). Lower PGRMC1 expression in HCC was significantly associated with higher serum alpha-fetoprotein expression ($P = 0.004$), poorer tumor differentiation ($P = 0.045$) and liver capsule penetration ($P = 0.038$). Low PGRMC1 expression was an independent predictor for worse disease-free survival ($P = 0.002$, HR = 2.384,

CI: 1.377-4.128) in our cases, as well as in the TCGA cohort ($P < 0.001$, HR = 2.857, CI: 1.781-4.584). The expression of PGRMC2 did not relate to patient outcome. PGRMC1 knockdown promoted a poorly differentiated phenotype and proliferation of HCC cells *in vitro*, while PGRMC1 overexpression caused the opposite effects.

CONCLUSION

PGRMC1 is a non-classical hormonal receptor that negatively regulates hepatocarcinogenesis. PGRMC1 down-regulation is associated with progression of HCC and is a poor prognostic indicator.

Key words: Progesterone receptor membrane component 1; Hormonal receptor; Proliferation; Hepatocellular carcinoma; Prognosis

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Core tip: Neither estrogen receptor or progesterone receptor are commonly expressed in hepatocellular carcinoma (HCC), implying the existence of other hormone-related events in the pathogenesis of HCC. Most primary HCC cases expressed progesterone receptor membrane component 1 (PGRMC1) (89.9%) in our clinical cohort ($n = 89$). Down-regulation of PGRMC1 was associated with poor tumor differentiation and worse patient survival. The potential prognostic significance was independently validated by The Cancer Genome Atlas (TCGA) database ($n = 373$). Knockdown of PGRMC1 promoted proliferation and a poorly differentiated phenotype *in vitro*. Overexpression of PGRMC1 resulted in suppressed proliferation in response to progesterone treatment. PGRMC1 is a prognostic marker and a potential auxiliary therapeutic target for human HCC.

Tsai HW, Ho CL, Cheng SW, Lin YJ, Chen CC, Cheng PN, Yen CJ, Chang TT, Chiang PM, Chan SH, Ho CH, Chen SH, Wang YW, Chow NH, Lin JC. Progesterone receptor membrane component 1 as a potential prognostic biomarker for hepatocellular carcinoma. *World J Gastroenterol* 2018; 24(10): 1152-1166 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i10/1152.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i10.1152>

INTRODUCTION

The major risk factors for hepatocellular carcinoma (HCC) are chronic liver diseases induced by hepatitis B (HBV) and hepatitis C (HCV), alcohol abuse, and non-alcoholic steatohepatitis. Regardless of etiology, the incidence of HCC is higher in males than in females, with a male to female ratio between 2:1 and 4:1^[1]. However, the biological role of sex hormones and their receptors in HCC remains poorly understood. The androgen

receptor appears to contribute to HCC development by acting as a tumor promoter^[2], whereas the estrogen receptor (ER) appears to act as a tumor suppressor^[3]. Oophorectomy performed during premenopausal years has been found to be a risk factor for HCC^[4]. The human liver is important in the metabolism of progesterone. Progesterone is known to inhibit autophagy and to augment epirubicin-induced apoptosis in hepatoma cells by increasing oxidative stress and upregulating Fas/FasL^[5,6]. In a clinical trial, HCC patients with variant ERs had a significantly longer median survival if megestrol acetate was given^[7], suggesting that progesterone is protective against HCC.

Progesterone receptor membrane component 1 (PGRMC1) and PGRMC2, which belong to the membrane-associated progesterone receptor (MAPR) family, have been suggested to be non-classical progesterone receptors^[8,9]. These proteins contain a cytochrome b5-like heme/steroid-binding domain. Both PGRMC1 and PGRMC2 are derived from a single gene. The structure of PGRMC1 contains two SH2 target sequences, a SH3 target sequence, a tyrosine kinase target site, two acidophilic kinase (CK2, casein kinase 2) target sites, and binding sites for ERK1 and PDK1. PGRMC2 differs from PGRMC1 in the following aspects: First, the transmembrane domain and N-terminals are different, resulting in diverse interaction partners in the lumen of sub-cellular organelles or on the cell membrane surface. Second, the SH3 target sequence of PGRMC1 with its consensus CK2 site is absent in PGRMC2, suggesting that PGRMC2 may not interact with SH3-containing proteins. Third, PGRMC2 has a predicted PDGFR or EGFR target and an additional potential CK2 site^[8]. Therefore, these two proteins may have different interacting partners in terms of connecting with the cellular membrane, organelles and cell signaling molecules.

In the case of tumorigenesis, PGRMC1 expression is associated with advanced-stage disease and poor prognoses in both breast and ovarian cancer^[10,11]. However, several studies have shown that PGRMC1 mediates the anti-mitotic actions of progesterone in endometrial and ovarian cancer cells^[12,13]. With regard to PGRMC2, copy number loss has been correlated with nodal metastasis in uterine cervical adenocarcinoma, suggesting that PGRMC2 can function as a metastasis suppressor^[14]. PGRMC2 negatively affects SKOV-3 ovarian cell migration^[15]. Therefore, PGRMC1 and PGRMC2 may have multiple biological functions related to metabolism and carcinogenesis. A proteomic study showed that PGRMC1 was expressed in HCC^[16] but there is no information regarding PGRMC1 or PGRMC2 expression patterns in HCC or their clinical significance in this disease. To address this issue, we examined PGRMC1 and PGRMC2 expression in a clinical cohort of paired HCC and non-tumor tissue samples ($n = 89$). We analyzed an independent HCC cohort ($n = 373$) from The Cancer Genome Atlas (TCGA cohort) to validate our findings. We also investigated the significance of

PGRMC1 in cell proliferation, differentiation, migration, and invasion *in vitro*.

MATERIALS AND METHODS

Patients and samples

Eighty-nine patients who underwent surgical resection for HCC at National Cheng Kung University Hospital (NCKUH) from January 1995 to September 2000 were included in this study. Frozen tissue, serum samples and archival paraffin blocks were retrieved from the Human Biobank at NCKUH. HCC differentiation was categorized according to the World Health Organization (WHO) system^[17]. Six paraffin samples and four frozen liver samples were retrieved from six normal non-hepatitis patients who underwent surgery for cavernous hemangioma and acted as controls.

Bioinformatic TCGA dataset analysis

We analyzed the TCGA provisional dataset (TCGA dataset, <https://tcga-data.nci.nih.gov/tcga/>), which contained 373 HCC patients with mRNA expression data (RNA Seq V2 RSEM), clinicopathological indicators, and follow-up information. Of these patients, 50 had expression data pertaining to HCC and matched adjacent non-tumor tissue samples. Disease-free survival (DFS) and overall survival (OS) were calculated based on PGRMC1 and PGRMC2 expression. Expression levels greater than the median were classified as high expression; otherwise, they were classified as low expression.

Western blotting

Protein lysates were prepared from either frozen tissue samples or HCC cell lines. Equal amounts of protein (50 micrograms) were separated *via* 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The proteins were then transferred to nitrocellulose membranes and stained with Ponceau S to assess transfer quality and ensure equal sample loading. The primary antibodies used were anti-PGRMC1 (Abnova Corporation, Walnut, CA, United States. PAB20135, 1:3000), anti-PGRMC2 (Abnova Corporation. H00010424-M04, 1:1000), anti-PR (Ventana Medical Systems, Inc. 790-2223, 1:100), anti-alpha-fetoprotein (Dako Cytomation, Inc., Carpinteria, CA, USA. A0008, 1:1000), anti-Glypican3 (BioMosaics, Burlington, VT, United States. 1G12, 1:1000) and anti- β -actin (GeneTex Inc. GTX109639, 1:10000). The indicated secondary antibodies (anti-rabbit and anti-mouse, IgG-HRP; Santa Cruz Biotechnology) were used to amplify the signals as appropriate.

Immunohistochemical staining and interpretation

Immunohistochemical staining was performed with primary antibodies against ER (Ventana Medical Systems, Inc., Arizona, United States), PR (Ventana

Medical Systems, Inc.), PGRMC1 (Abnova Corporation, Walnut, CA, United States. PAB20135) or PGRMC2 (Abnova Corporation. H00010424-M04). ER, PR, PGRMC1 and PGRMC2 expression was graded independently by two pathologists (Tsai HW and Ho CL) according to the percentages of stained hepatocytes or HCC cells. Because PGRMC1 is found in the cytosol and subcellular organelles^[8], cytoplasmic staining was considered to be positive. High PGRMC expression was defined as more than two-thirds of the cells exhibited positive staining. In the case of ER and PR, nuclear staining was considered to be positive.

In-gel trypsin digestion and mass spectrometry

Fifty micrograms of tissue extracts were resolved using 12% SDS-PAGE and were stained using Coomassie brilliant blue r-250. The protein spot located between 20–28 kDa was excised and then in-gel digested using 20 ng/ μ L of trypsin (Promega, San Luis Obispo, CA, United States, sequencing grade) in 10 mmol/L NH_4HCO_3 with an enzyme-to-substrate ratio of 1:100 at 37 °C overnight. Peptides were extracted using 50% acetonitrile in 1% formic acid followed by sonication.

A nanoflow high-performance liquid chromatography system (LC Packings, Amsterdam, The Netherlands) equipped with a C18 nano-precolumn cartridge (*i.d.* = 300 μ m \times 1 mm, 5 μ m C18, P/N160458; LC Packings) and a C18 column (*i.d.* = 75 μ m, o.d. = 280 μ m \times 15 cm, 3 μ m C18, LC Packings) was coupled online to a Q-TOF micro-instrument (Micromass, Manchester, United Kingdom). Mobile phase A was 0.1% formic acid in a 5% acetonitrile solution, and mobile phase B was 0.1% formic acid in 80% acetonitrile. A linear gradient from 5% to 90% B over 60 min at a flow rate of 250 nL/min was applied. A survey MS spectrum with a mass-to-charge ratio ranging from 400 to 1600 was followed by a MS/MS scan at a mass-to-charge ratio ranging from 50 to 2000. The threshold to switch from MS to MS/MS was 10 counts. Raw data were processed into peak lists, and then a Mascot search on a Swiss-Prot (human) protein database was conducted.

The mass tolerance was set at 0.2 Da for both the precursor and product ions. Dimethyl labeling of both the N-terminal and lysine residues was chosen for variable modifications; Carbamidomethyl (C) was chosen for fixed modification, and one miss cleavage on Arg-C was allowed. A cutoff value of 20 was set for the ion score to eliminate proteins with low matches. The default significance threshold for protein identification was set at $P < 0.05$.

Measurement of progesterone levels in sera and tissue

Tissue samples were minced and homogenized at a 1:10 (w:v) ratio with phosphate buffered saline at pH 7.4 using a homogenizer (PRO Scientific Inc., Oxford, CT, United States). The homogenates were centrifuged at $9000 \times g$ at 4 °C for 30 min and the supernatants were immediately analyzed. The progesterone levels in the sera and tissue supernatants were analyzed using

the Elecsys and Cobas e-immunoassay analyzer (Roche Diagnostics, Mannheim, Germany).

Cell lines

The HepG2 and Huh7 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Corp., Carlsbad, CA, United States) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μ g/mL) in a humidified incubator at 37 °C with 5% CO_2 , whereas the PLC/PRF/5 and Hep3B cell lines were maintained in modified Eagle's medium (MEM, Invitrogen Corp.) supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μ g/mL) in a humidified incubator at 37 °C with 5% CO_2 . Huh-7 cells were obtained from JCRB. Hep3B, HepG2 and PLC/PRF/5 cells were obtained from BCRC.

Knockdown and overexpression of PGRMC1

pLKO.1 plasmids expressing small hairpin RNA (shRNA) were purchased from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). Lentivirus particles were obtained from RNAi Core of the Research Center of Clinical Medicine, NCKUH. The following shRNAs were used to knock down PGRMC1 expression in HepG2 and Hep3B cells: sh-1: TRCN0000311671, target sequence: 5'-ACTGTGTACTCAGATGAGGAA-3'; sh-2: TRCN0000363644, target sequence: 5'-CGCCGACCCAAAGCGATCTGGA-3'; and sh-3: TRCN0000349346, target sequence: 5'-AGGATGAGTACGATGACCTT-3'. A pLKO_TRC005 plasmid was used as a negative control. PGRMC1 knockdown was performed as described previously^[15].

The pMSCVpuro (BD Clontech), pMSCV-PGRMC1, and pSUPERretro vectors were co-transfected into GP2-293T packaging cells along with VSV-G plasmids for 48 h using the calcium phosphate method. Either PLC/PRF/5 or Huh7 cells (1×10^6 cells/well) were seeded in a 6-cm dish and incubated overnight under 5% CO_2 at 37 °C. The retroviral supernatant was treated with 8 ng/mL polybrene (Sigma, St. Louis, MO, United States) to infect the cells. Pooled PLC/PRF/5 or Huh7 cells expressing either pMSCVpuro or pMSCV-PGRMC1 were selected using 0.7 μ g/mL puromycin (Sigma-Aldrich).

XTT proliferation assay

Stable pools were seeded in 24-well plates for 96 h. Triplicate wells were plated at each time point and examined at 24-h intervals over 4 d. The number of viable cells for each time point was determined using the XTT reagent, according to standard protocols (Roche Diagnostics GmbH, Vienna, Austria). To evaluate the impact of progesterone treatment, control cells and PGRMC1-overexpressing cells were cultured to 50% confluence and treated with progesterone (0, 10, 100, or 1000 nmol/L) for 48 h before the XTT assay.

In vitro migration and invasion assay

Transwell migration and invasion assays were performed using 24-well 8-micrometer pore Transwell plates,

Table 1 Prognostic significance of clinicopathological indicators, PGRMC1 and PGRMC2 for disease-free survival in the clinical cohort (*n* = 89)

Factor	Group	DFS univariate			DFS multivariate		
		HR	95%CI	<i>P</i> value	HR	95%CI	<i>P</i> value
Age, yr	< 60/≥ 60	0.997	0.609-1.632	0.989			
Sex	Male/female	1.014	0.599-1.715	0.960			
Viral infection				0.825			
	B/C	1.177	0.703-1.971				
	B/B + C	1.068	0.448-2.547				
Child-Pugh score	5/≥ 6	2.407	1.346-4.302	0.003 ^a	2.005	(1.097-3.665)	0.024 ^a
Cirrhosis	-/+	0.743	0.664-1.776	0.299			
Serum AFP	< 100/≥ 100 ng/ml	1.357	0.823-2.238	0.231			
Differentiation	W/M-P	1.682	0.943-2.999	0.078			
Multifocal tumor	-/+	0.985	0.514-1.888	0.965			
Satellite nodule	-/+	2.173	1.303-3.624	0.003 ^a			NS
Tumor size	< 5/≥ 5 cm	2.446	1.486-4.028	< 0.001 ^a			NS
Tumor capsular invasion	-/+	0.985	0.545-1.780	0.959			
Vascular invasion	-/+	2.551	1.553-4.190	< 0.001 ^a			NS
Liver capsule penetration	-/+	2.235	1.010-4.945	0.047 ^a			NS
Bile duct invasion	-/+	3.200	0.986-10.385	0.053			
Margin status, mm	≥ 1 / < 1	3.793	2.079-6.920	< 0.001 ^a	4.720	(2.458-9.063)	< 0.001 ^a
AJCC stage	I-II/ IIIA-C	2.907	1.738-4.861	< 0.001 ^a	3.262	(1.895-5.617)	< 0.001 ^a
PGRMC1	H/L	1.918	1.145-3.213	0.013 ^a	2.384	(1.377-4.128)	0.002 ^a
PGRMC2	H/L	1.377	0.798-2.379	0.251			
ER	-/+	0.528	0.128-2.173	0.376			
PR	-/+	0.777	0.243-2.484	0.670			

^a*P* < 0.05. DFS: Disease-free survival; AFP: Alpha-fetoprotein; AJCC: American Joint Committee on Cancer; H: High expression; L: Low expression.

according to the manufacturer's instructions (Corning, New York, NY, United States). A total of 5×10^4 cells in serum-free DMEM medium were split onto the upper Transwell chamber, the membrane of which was coated with (for invasion) or without (for migration) Matrigel (BD Biosciences, San Diego, CA, United States). The lower chamber was filled with DMEM containing 10% FBS as a chemoattractant. After incubation for 24 h at 37 °C, non-invading cells on the upper side of the chamber were removed from the surface of the membrane by scrubbing, and the membrane was fixed with 4% paraformaldehyde for 10 min. The cells on the membrane were stained using crystal violet solution and detected *via* microscopy. Mean cell numbers were calculated from five random fields.

Statistical analysis

The correlations between PGRMC1 expression, PGRMC2 expression, viral infection status, and clinicopathological indicators were assessed using the Wilcoxon rank sum test, the χ^2 test, or Fisher's exact test, as appropriate. Paired data were analyzed using paired Student's *t*-tests or the Wilcoxon signed-rank test. For the *in vitro* experiments, a Student's *t*-test was used for simple comparisons. Data are presented as the mean \pm SEM. DFS and OS were calculated using the Kaplan-Meier method, and the log-rank test was used to assess the differences between groups. A Cox proportional hazards regression model was used to measure the independence of different factors. A Cox regression was performed *via* a forward stepwise analysis, and only the prognostic variables that were significant in the

univariate analysis were included in the model. *P* values less than 0.05 were considered statistically significant.

RESULTS

Identification and analysis of PGRMC1 and progesterone expression in HCC

The HCC tissue extract was resolved using SDS-PAGE. The protein spot located between 20 to 28 kDa was in-gel trypsinized and subjected to mass spectrometry (MS). PGRMC1 was detected using MS (Supplementary Table 1). We subsequently examined the expression of PGRMC1 in 10 paired tumor and non-tumor liver samples (7 males and 3 females) using Western blotting. Another membrane-associated progesterone receptor family member, PGRMC2 was also examined for comparison. The age of the patients ranged from 57 to 77 years. Expression of PGRMC1 and PGRMC2 was lower in all HCC samples compared with the corresponding non-tumor liver samples (Figure 1A). Furthermore, both PGRMC1 and PGRMC2 were highly expressed in the normal liver as was the case in the non-tumor liver samples (Supplementary Figure 1A).

Progesterone expression was also evaluated in the HCC patients (*n* = 10). Serum progesterone levels (mean: 0.276 ng/mL for male patients and 0.167 ng/mL for female patients) were within the 5th-95th physical range in men (0.2-1.4 ng/mL) and postmenopausal women (0.1-0.8 ng/mL) (Figure 1D). However, progesterone levels in the HCC tissue were significantly lower than in the non-tumor liver tissue (*P* = 0.007, Figure 1B and C).

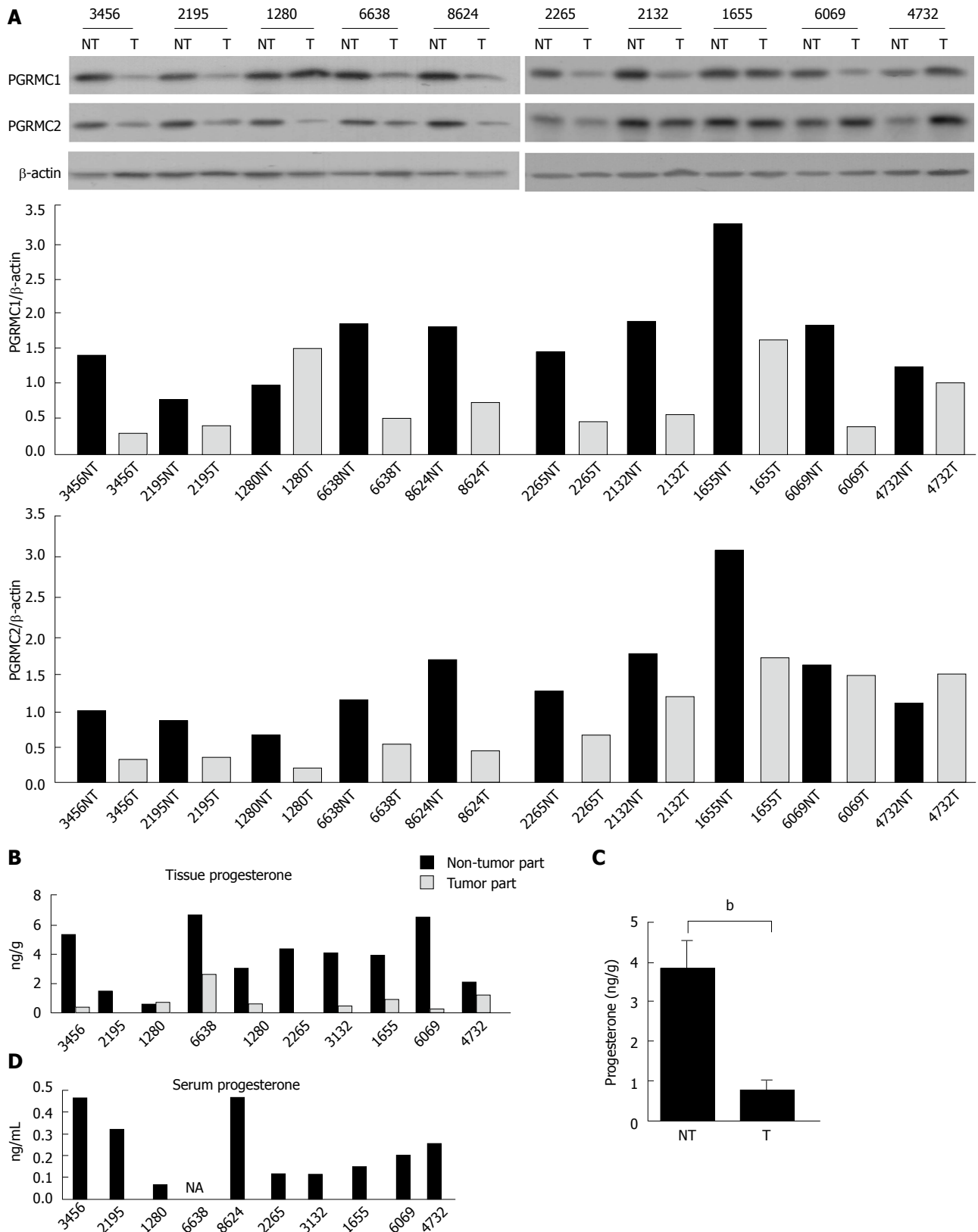


Figure 1 Western blot analysis of PGRMC1 and PGRMC2 expression in 10 paired hepatocellular carcinoma tumor (T)/non-tumor liver (NT) samples (A). B: Progesterone level in HCC tissue samples and non-tumor liver tissue; C: A comparison of progesterone levels in HCC (T) and non-tumor liver (NT) tissue samples; D: Serum progesterone level in the corresponding HCC patients. ^a*P* < 0.01.

Patient profiles

Immunohistochemical (IHC) staining for PGRMC1 and PGRMC2 was performed in the clinical cohort of 89 paired HCC and non-tumor liver samples. The profiles

of the 89 patients are summarized in Supplementary Table 2. The patient population included 65 men and 24 women. The mean age was 55.2 years. The mean follow-up duration was 44.5 mo (range, 0.9-133.1

Table 2 Prognostic significance of clinicopathological indicators, *progesterone receptor membrane component 1* and *PGRMC2* for disease-free survival in The Cancer Genome Atlas cohort ($n = 373$)

Factor	Group	DFS univariate			DFS multivariate		
		HR	95%CI	P value	HR	95%CI	P value
Age, yr	< 60/≥ 60	1.019	0.756-1.373	0.903			
Sex	Male/female	1.157	0.845-1.585	0.364			
HCC risk factors				0.014 ^a			< 0.001 ^a
	B/NAFLD	1.433	0.601-3.414	0.417	2.903	(1.130-7.461)	0.027 ^a
	B/Alcohol	1.449	0.948-2.217	0.087	1.002	(0.595-1.689)	0.993
	B/C	2.421	1.422-4.121	0.001 ^a	3.368	(1.773-6.395)	< 0.001 ^a
Child-Pugh score	A/B-C	1.332	0.709-2.504	0.372			
Cirrhosis	-/+	1.117	0.761-1.641	0.572			
Serum AFP, ng/ml	< 100/≥ 100	1.097	0.752-1.599	0.632			
Tumor grade	2/4/2001	1.330	0.868-2.038	0.191			
Vascular invasion	-/+	2.001	1.418-2.823	< 0.001 ^a	3.040	(1.889-4.891)	< 0.001 ^a
Residual tumor	-/+	1.733	0.938-3.200	0.079			
AJCC stage	I - II / III - IV	2.354	1.693-3.272	< 0.001 ^a	1.899	(1.107-3.255)	0.020 ^a
PGRMC1	H/L	1.834	1.359-2.475	< 0.001 ^a	2.857	(1.781-4.584)	< 0.001 ^a
PGRMC2	H/L	1.242	0.923-1.671	0.152			

^a $P < 0.05$. DFS: Disease-free survival; NAFLD: Non-alcoholic fatty liver disease; AFP: Alpha-fetoprotein; AJCC: American Joint Committee on Cancer; H: High expression; L: Low expression.

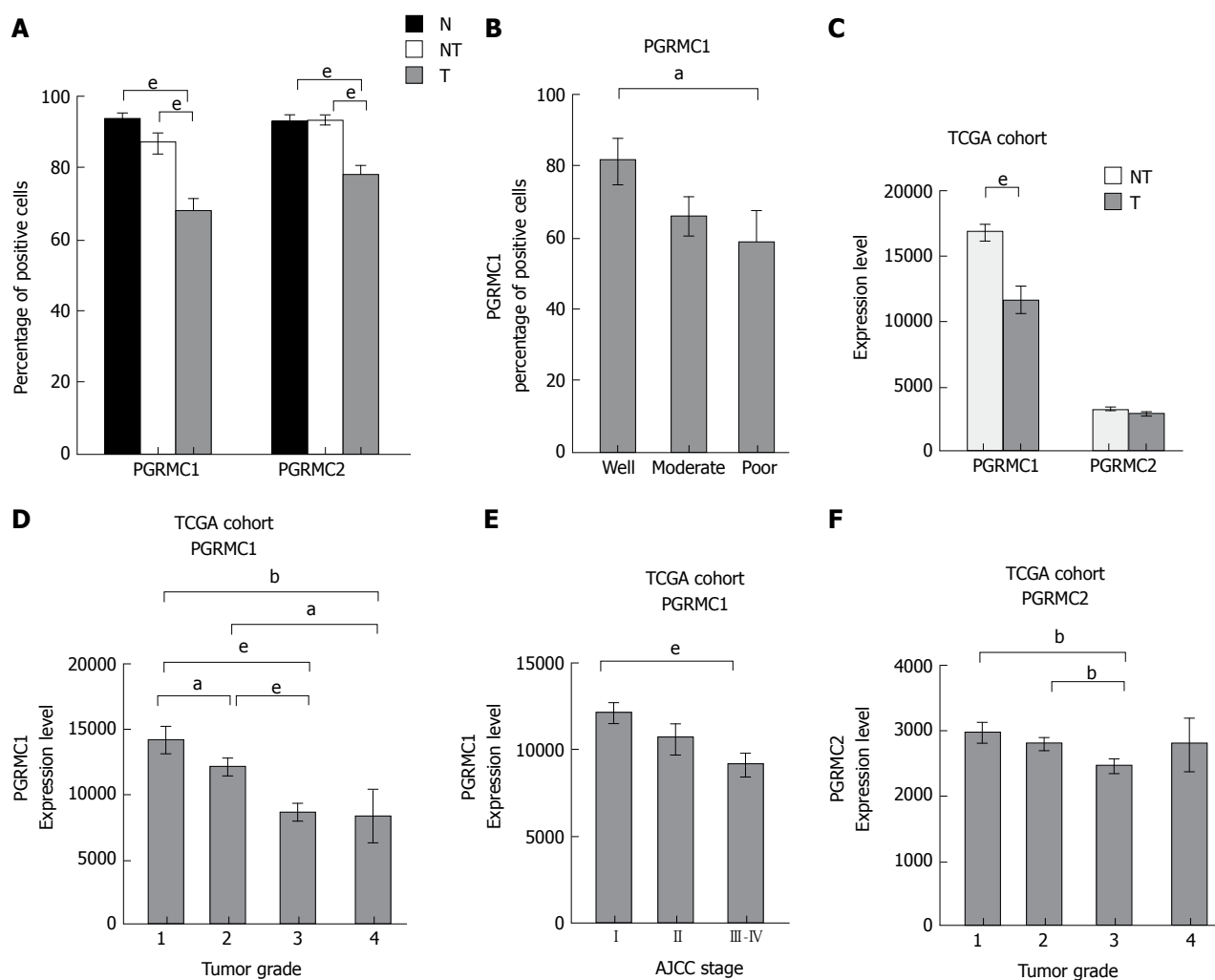


Figure 2 PGRMC1 and PGRMC2 expression levels, expressed as positive immunohistochemical staining percentages in the clinical cohort (A and B) and as normalized mRNA expression in the TCGA cohort (C-F). A: A comparison of PGRMC1 and PGRMC2 staining in HCC (T), non-tumor liver (NT) and normal liver (N) tissue samples; B: A comparison of PGRMC1 staining in HCC samples with different degrees of tumor differentiation; C: A comparison of PGRMC1 and PGRMC2 mRNA expression levels in HCC (T) and non-tumor liver (NT) samples; D: A comparison of PGRMC1 mRNA expression levels in HCC samples with different tumor grades; E: A comparison of PGRMC1 mRNA expression levels in HCC samples with different tumor stages; F: A comparison of PGRMC2 mRNA expression levels in HCC samples with different tumor grades. ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$.

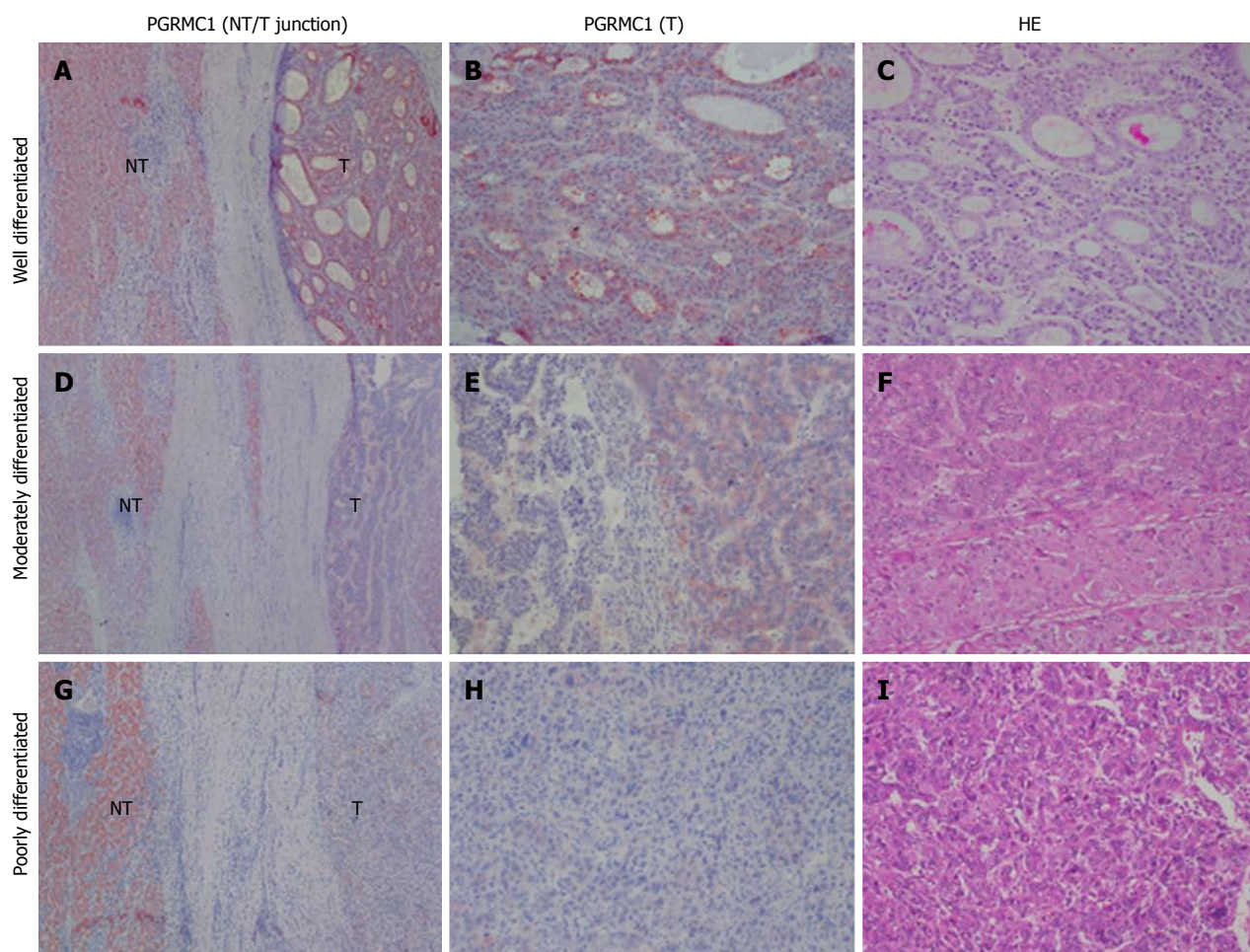


Figure 3 Representative images of PGRMC1 immunohistochemical staining in hepatocellular carcinoma. A-C: Well-differentiated HCC; D-F: Moderately differentiated HCC; G-I: Poorly differentiated HCC. Note that higher PGRMC1 expression was observed in non-tumor liver tissue samples (NT) as compared to HCC tissue samples (T) (A, D and G) and a proportion of HCC cells showed loss of PGRMC1 staining in (E) (A, D and G: 40 \times ; B, C, E, F, H, and I: 100 \times).

mo). Sixty-seven patients (75.3%) developed local recurrence at a mean of 21.8 mo after surgery (range, 0.2-115.1 mo). A total of 45 patients (50.1%) died of HCC after having survived for a mean of 35.4 mo after surgery (range, 1.3-123.4 mo). The profiles of the 373 patients in the TCGA cohort are summarized in Supplementary Table 3.

The association of PGRMC1 and PGRMC2 expression with clinicopathological indicators

Few HCC cases expressed ER or PR (5.6% and 4.5%, respectively) (Supplementary Figure 2A-C). The mean cell percentage of ER expression was lower in the HCC tissue than in the non-tumor tissue (mean: 0.7% vs 12.4%, $P < 0.001$) (Supplementary Figure 2D). The mean cell percentage of PR expression was very low in the HCC tissue and non-tumor tissue (mean: 0.12% vs 0.01%, $P = 0.172$) (Supplementary Figure 2E). Positive IHC staining for PGRMC1 and PGRMC2 was observed in 80 (89.9%) and 89 (100%) cases of HCC, respectively. PGRMC1 (mean: 67.9% vs 86.9%, $P < 0.001$) and PGRMC2 (mean: 77.3% vs 92.6%, $P < 0.001$) were expressed at lower percentages in

tumor cells than in non-tumor cells (Figure 2A, and 3, and Supplementary Figure 3). IHC expression of PGRMC1 and PGRMC2 in the normal liver tissue was not significantly different from that in the non-tumor liver samples, a result consistent with the Western blot experiment (Figure 2A and Supplementary Figure 1B and 1C). The PGRMC1 and PGRMC2 expression levels were compared among the normal livers of healthy persons, non-cirrhotic livers, and cirrhotic livers of HCC patients (Supplementary Figure 4A and B). There were no significant differences between the liver samples of healthy persons and those of HCC patients. In both non-cirrhotic patients and cirrhotic patients, the PGRMC1 and PGRMC2 expression levels were downregulated in the HCC tissue compared to non-tumor liver tissue (Supplementary Figure 4C-F). In the TCGA cohort, the HCC tissue samples exhibited lower *PGRMC1* mRNA expression levels than in the non-tumor tissue samples ($P < 0.001$), while *PGRMC2* levels between the HCC and non-tumor livers were not significantly different (Figure 2C).

Lower PGRMC1 expression in HCC was associated with poor HCC differentiation ($P = 0.045$) (Figure 2B

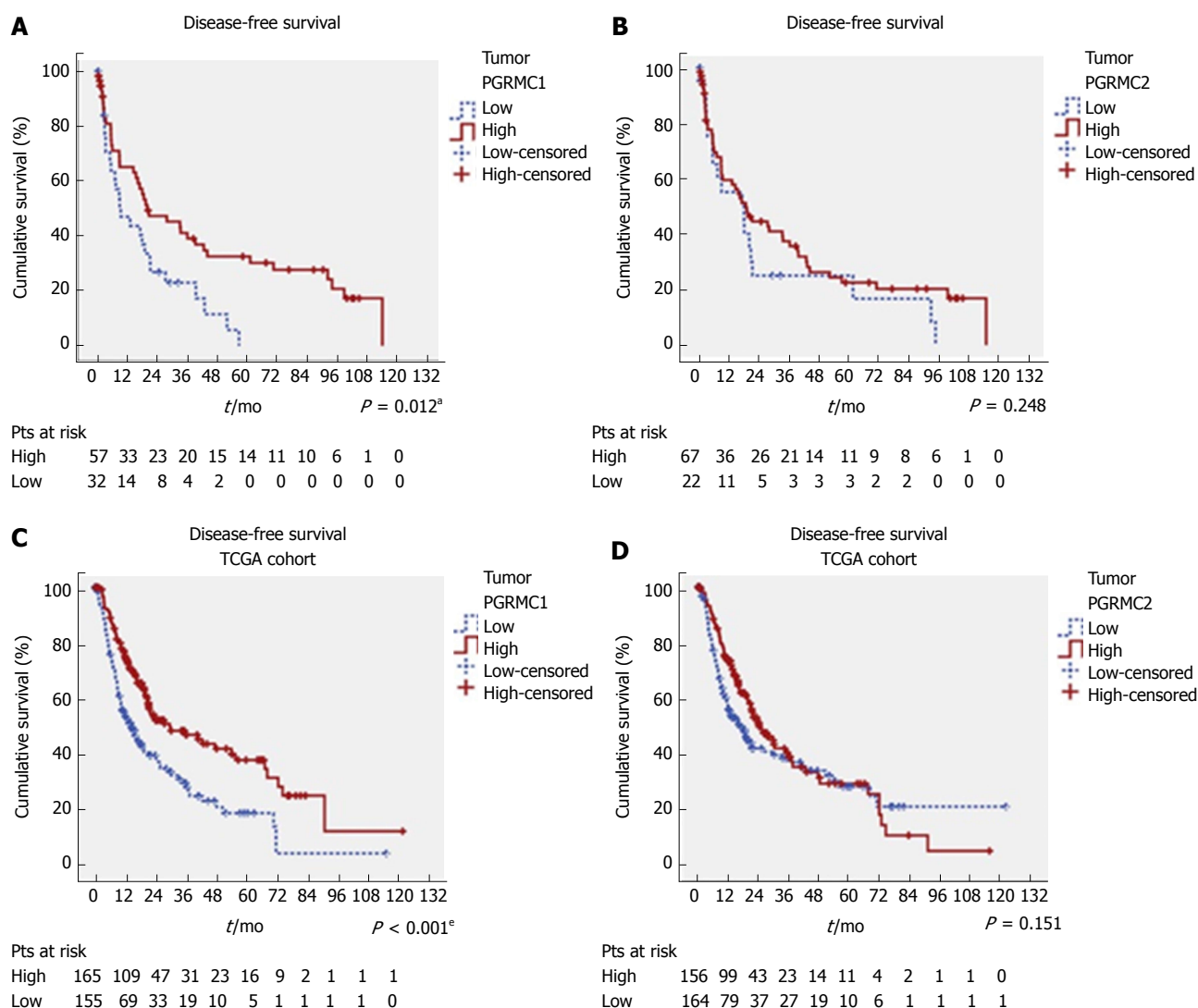


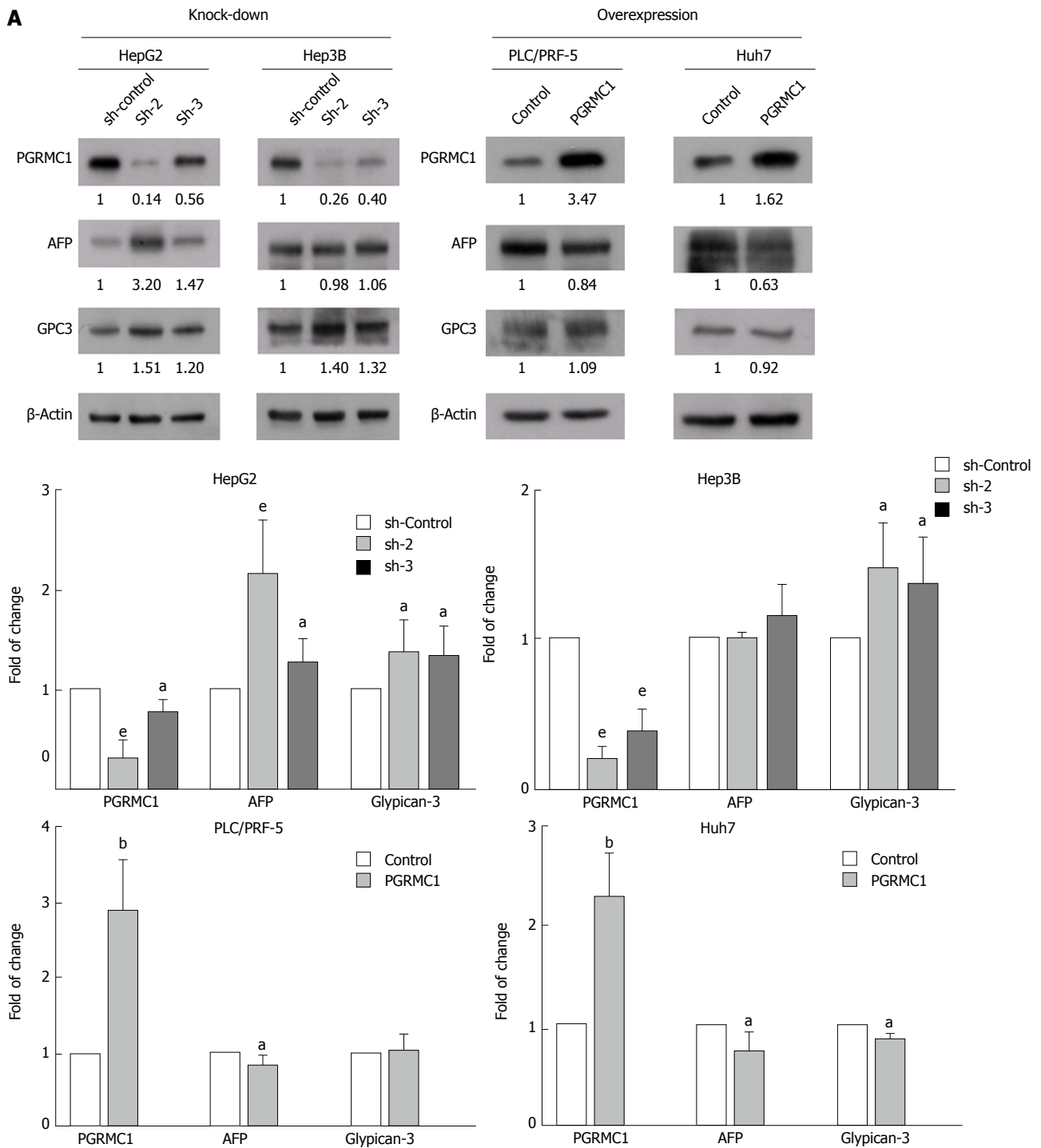
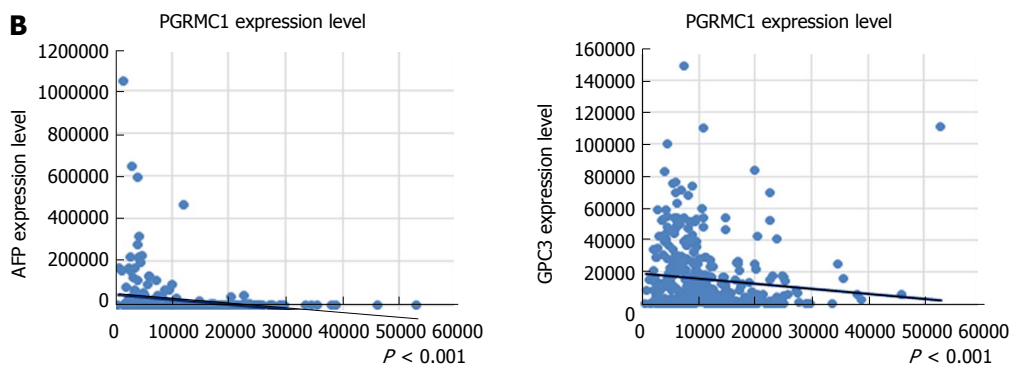
Figure 4 Kaplan-Meier analysis of the relationships of PGRMC1 and PGRMC2 expression with disease-free survival (DFS) in the clinical cohort (A-B) and TCGA cohort (C-D). ^a $P < 0.05$, ^e $P < 0.001$.

and Figure 3), younger age ($P = 0.016$), higher serum alpha-fetoprotein (AFP) levels ($P = 0.004$), and liver capsule penetration ($P = 0.038$) (Supplementary Table 4). Lower PGRMC2 expression in HCC was significantly associated with tumor capsular invasion ($P = 0.048$), liver capsule penetration ($P = 0.035$), and bile duct invasion ($P = 0.032$) (Supplementary Table 4). In the TCGA cohort, lower PGRMC1 expression in HCC was associated with higher tumor grade ($P < 0.001$) (Figure 2D), younger age ($P = 0.014$), female gender ($P = 0.015$), higher serum AFP expression ($P < 0.001$), vascular invasion ($P = 0.007$), and higher AJCC stage (Figure 2E) ($P = 0.002$) (Supplementary Table 5). Lower PGRMC2 expression in HCC was significantly associated with higher tumor grade ($P = 0.022$) (Figure 2F) and younger age ($P = 0.006$) (Supplementary Table 5). Female patients exhibited higher baseline non-tumor liver tissue PGRMC1 expression in the TCGA cohort ($P < 0.001$) (Supplementary Figure 5A-D). Using corresponding non-tumor liver tissue samples as a reference, it was determined that female HCC

patients in the TCGA cohort exhibited greater PGRMC1 down-regulation than male patients (Supplementary Figure 5E-H).

Prognostic significance of PGRMC1 and PGRMC2 in HCC

Low IHC PGRMC1 expression in HCC was significantly associated with worse DFS ($P = 0.012$) (Figure 4A), but was not significantly associated with OS ($P = 0.395$) (Supplementary Figure 6A). In contrast, PGRMC2 expression was not correlated with survival status (Figure 4B and Supplementary Figure 6B). The univariate analysis showed that the Child-Pugh scores ($P = 0.003$), satellite lesions ($P = 0.003$), tumor size ($P < 0.001$), vascular invasion ($P < 0.001$), liver capsule perforation ($P = 0.047$), insufficient surgical margins ($P < 0.001$), AJCC stage ($P < 0.001$), and low PGRMC1 expression ($P = 0.013$) were significant predictors of worse DFS (Table 1). The multivariate analysis showed that the Child-Pugh scores ($P = 0.024$, HR = 2.005, CI: 1.097-3.665), surgical margins ($P < 0.001$, HR =

A**B**

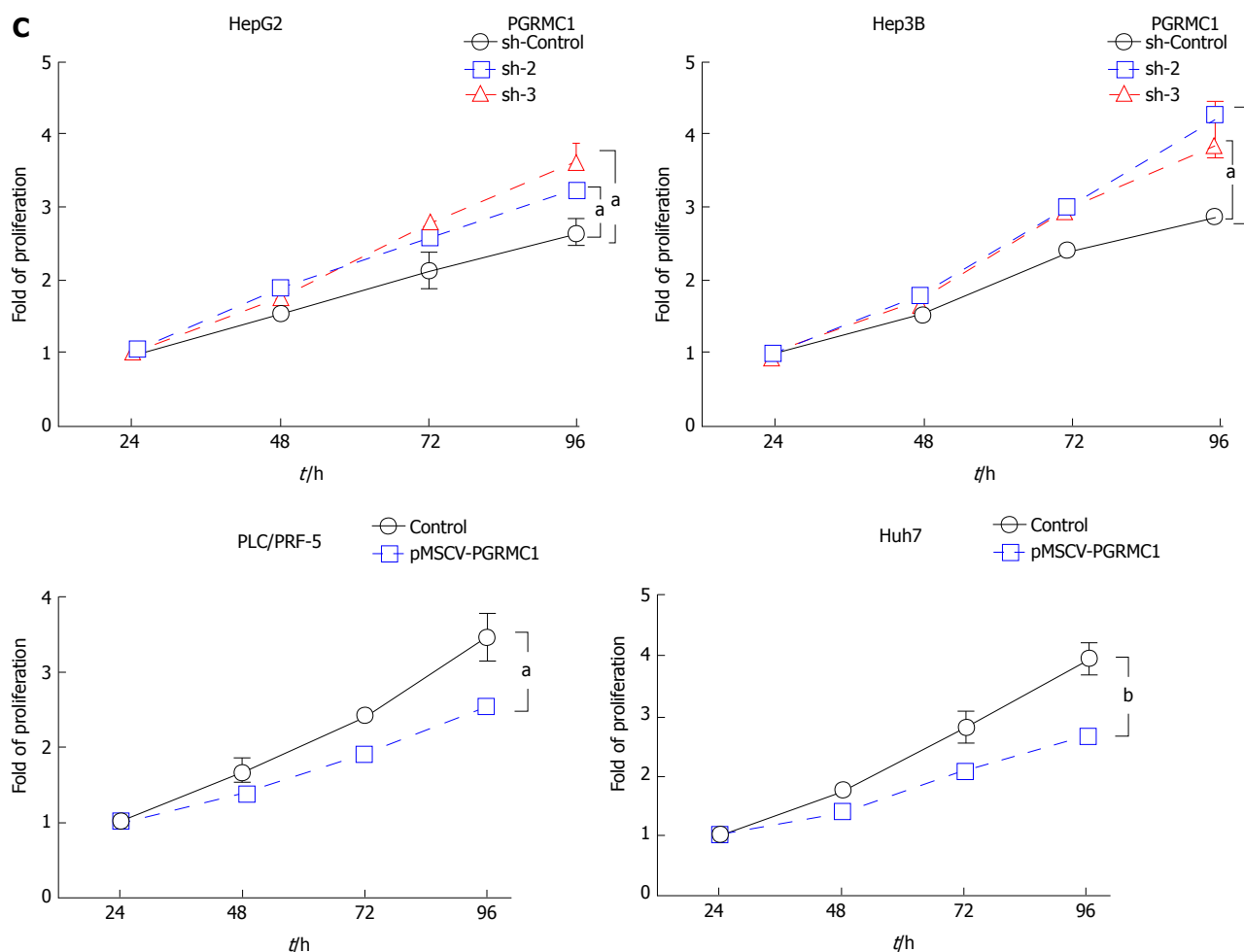


Figure 5 Alpha-fetoprotein and glypican-3 expression in PGRMC1-knockdown HepG2 and Hep3B cells and PGRMC1-overexpressing PLC/PRF/5 and Huh7 cells (A). B: The correlation of PGRMC1 with AFP and GPC3 in HCC tissue samples in the TCGA cohort; C: Cell proliferation assay (XTT) of PGRMC1-knockdown HepG2 and Hep3B cells and PGRMC1-overexpressing PLC/PRF/5 and Huh7 cells. The experiment was performed in triplicate. ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$. AFP: Alpha-fetoprotein; GPC3: Glypican-3.

4.720, CI: 2.458-9.063), AJCC stage ($P < 0.001$, HR = 3.262, CI: 1.895-5.617), and low PGRMC1 expression ($P = 0.002$, HR = 2.384, CI: 1.377-4.128) were independently associated with DFS (Table 1).

To validate our findings, we analyzed the TCGA cohort data. Patients with low PGRMC1 expression consistently exhibited worse DFS ($P < 0.001$) and OS than those with high PGRMC1 expression ($P = 0.013$), as determined by the Kaplan-Meier and log-rank test analyses (Figure 4C and Supplementary Figure 6C). PGRMC2 expression was not correlated with survival status (Figure 4D and Supplementary Figure 6D). The univariate analysis showed that HCC risk factors ($P = 0.014$), vascular invasion ($P < 0.001$), AJCC stage ($P < 0.001$), and low PGRMC1 expression ($P < 0.001$) were significant predictors of worse DFS (Table 2), and that AJCC stage ($P < 0.001$) and low PGRMC1 expression ($P = 0.014$) were significant predictors of worse OS (Supplementary Table 6). The multivariate analysis showed that low PGRMC1 expression was an independent predictor of both worse DFS ($P < 0.001$, HR = 2.857, CI: 1.781-4.584) and worse OS ($P =$

0.020, HR = 1.556, CI: 1.072-2.260) (Table 2 and Supplementary Table 6).

Effects of PGRMC1 on proliferation, differentiation, migration and invasion of HCC cells

As PGRMC1 was significantly associated with HCC prognosis, we examined its biological significance *in vitro*. HepG2 and Hep3B cells exhibited higher PGRMC1 expression than PLC/PRF/5 and Huh7 cells. Therefore, we knocked down PGRMC1 in HepG2 and Hep3B cells and overexpressed PGRMC1 in PLC/PRF/5 and Huh7 cells (Figure 5A). Both AFP and glypican-3 (GPC3) are well-known oncofetal proteins in malignant transformation and dedifferentiation of HCC. Higher expression of these markers has been associated with poor differentiation of HCC^[18,19]. Knockdown of PGRMC1 resulted in increased expression of AFP and GPC3 in HepG2 cells, while GPC3 expression was increased in Hep3B cells (Figure 5A). In contrast, overexpression of PGRMC1 suppressed expression of AFP in PLC/PRF/5 cells and suppressed expression of AFP and GPC3 in Huh7 cells (Figure 5A). In addition, PGRMC1

expression was inversely correlated with AFP and GPC3 expression in the HCC tissue samples (Figure 5B) and serum AFP levels (Supplementary Tables 4 and 5). PGRMC1 knockdown resulted in significantly increased proliferation of HepG2 and Hep3B cells (Figure 5C). In contrast, PGRMC1 overexpression resulted in decreased proliferation of PLC/PRF/5 and Huh7 cells (Figure 5C). PGRMC1 did not have significant effects on cell migration and invasion (Supplementary Figure 7).

As PGRMC1 was downregulated in HCC and it has been suggested that it mediates the anti-proliferative effects of progesterone^[12,13], we assessed if overexpression of PGRMC1 in HCC cells could increase the anti-proliferative effect under progesterone treatment. PGRMC1 overexpression resulted in a significant decrease in the proliferation of PLC/PRF-5 cells and Huh7 cells in response to progesterone treatment (10^{-6} mol/L) (Supplementary Figure 8A and B). Expression of PR was not increased in these cells (Supplementary Figure 8C). On this basis, it is suggested that a more plausible explanation for the significant progesterone treatment effect on PGRMC1-overexpressing PLC/PRF-5 and Huh7 cells may be the overexpression of PGRMC1 *per se* rather than upregulated PR in these cells.

DISCUSSION

In this study, few HCC cases expressed ER and PR (5.6% and 4.5%, respectively), implying that an alternative hormone-related event may be involved in this sexually dimorphic malignancy. Both PGRMC1 and PGRMC2 were expressed in normal liver and non-tumor liver, but were down-regulated in HCC. In addition, the level of progesterone was lower in the HCC as compared to the non-tumor liver, suggesting that progesterone-related signaling is down-regulated in the pathogenesis of HCC. As down-regulated PGRMC1 and PGRMC2 were correlated with poorer differentiation and higher tumor grading, PGRMC down-regulation may play an important role in the progression of hepatocarcinogenesis. PGRMC1 differs from PGRMC2 in the transmembrane domain, N-terminals and SH3 target sequence, resulting in different interaction partners in terms of connecting with the cellular membrane, organelles and cell signaling molecules^[8]. PGRMC1 has been proposed as a sigma-2 receptor with capability to inhibit tumor growth^[20-22]. In our study, PGRMC1 was inversely associated with AFP and AJCC stage compared with PGRMC2. In multivariate analysis, PGRMC1 was an independent parameter in predicting better patient survival in two different cohorts. Therefore, only PGRMC1 was proved to be a prognostic biomarker in HCC. All of our patients had HBV and/or HCV infections, while more than half of the patients in the TCGA cohort had alcoholic and/or non-alcoholic fatty liver disease, suggesting that the findings of this investigation are universal. Together, PGRMCs, especially PGRMC1, may play a protective role in liver tumorigenesis.

Experiments *in vitro* demonstrated that PGRMC1 knockdown results in a poorly differentiated phenotype of HCC cells and increased proliferation. PGRMC1 contains two SH2 target sequences, an SH3 target sequence, a tyrosine kinase target site, two acidophilic kinase target sites, and ERK1 and PDK1 consensus binding sites^[8]. A prior report showed that PGRMC1 can interact with beta-tubulin and inhibit mitosis by increasing mitotic spindle stability^[13]. PGRMC1 protein has also been proposed as a sigma-2 receptor binding site^[20], and activation of the sigma-2 receptor can inhibit tumor growth^[21,22]. Thus, PGRMC1 is speculated to inhibit HCC progression through activation of hormone-independent signaling events.

PGRMC1 contains a motif common to heme-binding proteins, suggesting a role in oxidative metabolism^[23]. Free heme, *i.e.*, heme not appropriately bound by hemoproteins or heme-binding proteins, is a powerful pro-oxidant agent and therefore potentially toxic^[24]. Heme-binding proteins are required to maintain cellular stasis and to detoxify cells. Excessive heme iron has been reported to increase the risk of several types of cancer, such as colon cancer^[25], gastric cancer^[26], esophageal cancer^[27], and HCC^[28]. As a heme-binding protein, PGRMC1 has been reported to interact with P450 proteins and protect cells from DNA damage^[29-31]. Therefore, PGRMC1 may protect hepatocytes from oxidative stress and suppress carcinogenesis by appropriate heme delivery or heme containment.

Most prior PGRMC1 studies have been focused on the female genital organs or female-related cancers. PGRMCs are involved in regulating the menstrual cycle and ovarian granulosa cell function by acting as progesterone receptors^[9,32]. Endometrial expression of PGRMC1 in menstrual cycling is most abundant during the proliferative phase, while expression of PGRMC2 is highest during the secretory phase. These results highlight the differences between PGRMC1 and PGRMC2 in response to steroid hormones^[32]. Previous reports showed that PGRMC1 mediates the anti-mitotic actions of progesterone in endometrial and ovarian cancer cells^[12,13,33]. In immortalized granulosa cells, PGRMC1 suppresses cell cycle entry by binding to the GTPase activating protein binding protein 2^[33]. PGRMC1 also suppresses the T-cell-specific transcription factor/lymphoid enhancer factor (Tcf/Lef) and its downstream c-myc activity in ovarian granulosa cells^[9]. In this investigation, we provide evidence that overexpression of PGRMC1 may also activate the non-classical PR pathway in tumorigenesis. The potential of PGRMC1 being an alternative target for auxiliary anti-HCC treatment deserves further investigation.

Previous studies have shown that high levels of progesterone can be observed in patients with cirrhosis^[34]. This is likely due to impairment of progesterone metabolism in the liver. It is controversial whether high levels of progesterone are associated with premalignant cirrhosis. Previous studies have shown that the occurrence of natural menopause at a younger

age or oophorectomy performed at age 50 or younger is associated with increased risk of HCC^[4,35]. PR expression in HCC has been correlated with a better prognosis^[36]. These findings suggest that progesterone may be protective against HCC. Furthermore, progesterone can serve as the precursor for the major steroid hormones (androgens, estrogens, and corticosteroids). The oncogenic effects of androgen and the protective effects of estrogen and progesterone in liver may also depend on the hormonal receptors expressed on hepatocytes or cancer cells^[4].

Sex hormones can exert different tumorigenic properties depending on the tissue type. For example, contrary to their hypothetical protective role in liver cancer development, chronic exposure to estrogens favors carcinogenesis in the breast and uterus. Expression of PGRMC1 was shown to be upregulated in breast cancer and ovarian cancer and was found to be associated with advanced stage or poor prognosis^[10,11]. Its expression was more often detected in ER-negative breast cancers^[10], and it may act *via* cross-talk with nuclear or extranuclear ER receptors^[37]. PGRMC1 has been localized in hypoxic areas of breast cancer^[10] and demonstrated to activate the expression of vascular endothelial growth factor in glial cells^[38]. A D120G mutant of PGRMC1 increased the susceptibility of breast cancer cells to doxorubicin and camptothecin treatment^[39]. However, PGRMC1 has been reported to be associated with EGFR in lung cancer cells and to enhance susceptibility to the EGFR inhibitor, erlotinib^[40]. Overexpression of PGRMC1 in the MCF-7 breast cancer cell line sensitizes cancer cells to hydrogen peroxide treatment with corresponding hyperphosphorylation of Akt and IκB proteins^[29]. These findings suggest that PGRMC1 plays a plethora of biological roles in human cancers. In contrast to breast and ovarian cancer, PGRMC1 is downregulated in HCC. PGRMC1 is located on chromosome Xq22-q24. A prior genomic study found a frequent loss of heterozygosity of Xq (43%) in HCC^[41] with a progressive increase in fractional allelic imbalance from cirrhotic nodules at progressive stages (11%-57%) to HCC, suggesting its involvement in hepatocarcinogenesis. Furthermore, let-7/miR-98 was reported to repress PGRMC1^[42,43], and gradually elevated miR-98 has been associated with the progression of liver cancer^[44]. Therefore, microRNA could be an alternative regulatory mechanism in suppression of PGRMC1 expression. Further study is needed to clarify the mechanisms of PGRMC downregulation in HCC.

Overall, men are two to four times more likely to develop HCC than women^[1]. Estrogen has been shown to inhibit IL-6 production^[45]. Foxa1/a2 may interact with either the ER or AR to activate different hepatocyte target genes^[46]. HBx can increase AR N-terminal transactivation domain activation through c-Src kinase and enhance AR dimerization by inhibiting GSK-3 activity^[47]. This information may explain, in part, the

molecular mechanisms underlying gender differences in HCC development. Female patients in the current study exhibited higher baseline non-tumor liver tissue *PGRMC1* mRNA expression and greater HCC *PGRMC1* down-regulation than male patients, suggesting that greater PGRMC1 down-regulation is needed to induce HCC transformation in female patients, thus causing the gender disparities associated with HCC development.

In conclusion, expression of PGRMC1 and PGRMC2 was suppressed in HCC, and PGRMC1 down-regulation promoted HCC progression. PGRMC1 may play a protective role in hepatocarcinogenesis by inhibiting cell proliferation and tumor dedifferentiation. Further study is necessary to evaluate the potential of targeting PGRMC1 in HCC treatment.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is a sexually dimorphic disease with a significantly higher incidence in males than females. The androgen receptor appears to function as a tumor promoter, whereas the estrogen receptor appears to act as a tumor suppressor for HCC. Whether additional hormone-related events are implicated in the pathogenesis of HCC remain to be clarified.

Research motivation

The membrane-associated progesterone receptors, *i.e.* PGRMC1 and PGRMC2, have been investigated in female cancers of the breast, endometrium and ovary. PGRMC1 is thought to coordinate non-classical progesterone signaling. PGRMC1 was demonstrated to mediate the anti-mitotic actions of progesterone in endometrial and ovarian cancer cells. This study was performed to examine the significance of PGRMC1 and/or PGRMC2 in the progression of HCC.

Research objectives

The aim of this study was to clarify the potential significance of PGRMCs as prognostic biomarkers in HCC and their biological effects *in vitro*.

Research methods

Immunohistochemical staining of the estrogen receptor (ER), progesterone receptor (PR), PGRMC1 and PGRMC2 was performed in a clinical cohort consisting of 89 cases of paired HCC and non-tumor liver. The clinical implications of PGRMCs in HCC ($n = 373$) from The Cancer Genome Atlas (TCGA) database were *also analyzed*. The expression of PGRMC1 and PGRMC2 was correlated with clinicopathological indicators and the clinical outcome of HCC patients. The impact of PGRMC1 on the biological effects of HCC was investigated by knocking down its expression in HepG2 and Hep3B cell lines, and overexpressing in PLC/PRF-5 and Huh7 cell lines. The analyzed cellular functions included proliferation, differentiation, migration, and invasion.

Research results

Primary HCC demonstrated a high incidence of PGRMC1 (89.9%) and PGRMC2 (100%) expression, respectively. Down-regulated PGRMC1 was significantly associated with higher serum alpha-fetoprotein levels, poor tumor differentiation, liver capsule penetration, and the risk of recurrence. Low PGRMC1 expression was an independent indicator of worse disease-free survival. Knock-down of PGRMC1 promoted a poorly differentiated phenotype and proliferation of HCC *in vitro*, while over-expression of PGRMC1 suppressed cell proliferation.

Research conclusions

PGRMC1 is a prognostic marker for HCC. PGRMC1 may play a protective role in hepatocarcinogenesis by inhibiting cell proliferation and tumor

dedifferentiation.

Research perspectives

PGRMC1 could be a novel therapeutic target for human HCC, especially as a biotarget of chemoprevention.

ACKNOWLEDGEMENTS

We are grateful for the support from the Human Biobank, Research Center of Clinical Medicine and the Cancer Data Bank of National Cheng Kung University Hospital.

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P- Reviewer: Elalfy H, Guan YS, Hashimoto N **S- Editor:** Gong ZM
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World Journal of Gastroenterology
Volume 24 Number 10 March 14, 2018

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World Journal of Gastroenterology (*WJG*) is now indexed in Current Contents[®]/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch[®]), Journal Citation Reports[®], Index Medicus, MEDLINE, PubMed, PubMed Central and Directory of Open Access Journals. The 2018 edition of Journal Citation Reports[®] cites the 2016 impact factor for *WJG* as 3.365 (5-year impact factor: 3.176), ranking *WJG* as 29th among 79 journals in gastroenterology and hepatology (quartile in category Q2).

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NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

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PUBLICATION DATE
March 14, 2018

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ISSN 1007-9327



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