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PNMA1, regulated by miR-33a-5p, promotes proliferation and EMT in hepatocellular carcinoma by activating the Wnt/β-catenin pathway

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors throughout the world. However, its mortality rate remains very high due to the absence of early diagnostic modalities and effective treatments, and its specific pathogenesis remains unclear. Here, we investigated the role of PNMA1 in the tumorigenesis of HCC. We found that PNMA1 was significantly upregulated in HCC. Clinically, higher expression of PNMA1 was associated with aggressive phenotypes and poor prognosis. Functionally, silencing of PNMA1 repressed proliferation in vitro and in vivo, and knockdown of PNMA1 suppressed tumor cell migration and invasion. Via GSEA analysis, we predicted that PNAM1 may be related to the epithelial-mesenchymal-transition and the Wnt signaling pathway. Both these assumptions were confirmed in our study. Furthermore, we proved that miR-33a-5p participated in the posttranscriptional regulation of PNMA1. Together, our findings suggested that PNMA1 participated in HCC progression and may be a potential biomarker and therapeutic target for HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death in the world [1–3]. HBV and HCV infection were regarded as the main causes of HCC [4]. Current treatments for HCC include early surgical resection, radiotherapy and chemotherapy [5]. Unfortunately, patients diagnosed with HCC usually have poor survival, typically only 3–6 months, mostly because of late diagnosis and the lack of effective nonsurgical treatments [6]. Therefore, finding new targets for early diagnosis and treatment of HCC is a common goal.

PNMA1 is a member of the paraneoplastic Ma family (PNMA), consisting of at least fifteen family members [7]. Studies of the PNMA family members primarily focused on PNMA1-3, all of which had a close association with paraneoplastic disorder (PND), and on MOAP-1, mediating apoptotic signaling by interacting with Bax [7–9]. Chen et al. reported that PNMA1 was a proapoptotic protein in mouse neurons [10]. In addition, Jiang et al. demonstrated that PNMA1 served as an oncogene in human pancreatic ductal adenocarcinoma via the activation of the PI3K/AKT and MAPK/ERK pathways [11]. However, the role of PNMA1 in malignant tumors remains largely unknown, including HCC.

MicroRNAs (miRNAs) are small, noncoding RNAs (20–24 nt) that are key posttranscriptional regulators of gene expression; they directly interact with the 3′-untranslated region or 5′-untranslated region of target mRNAs [12,13]. Several lines of evidence have indicated that microRNAs participated in the development of tumors such as HCC [14–16].

In this study, we found that PNMA1 was upregulated in HCC tissues and that higher expression of PNMA1 was significantly associated with poor outcomes. Furthermore, we found that PNMA1 promoted cell proliferation and EMT progression via activation of the Wnt/β-catenin pathway and that miR-33a-5p participated in the posttranscriptional regulation of PNMA1.

2. Materials and methods

2.1. Patients and clinical specimens

A total of 70 pairs of HCC and corresponding adjacent nontumor specimens were simultaneously obtained from 70 HCC patients in Zhongnan Hospital, Wuhan University. All samples were stored with RNA protective reagent at −80 °C. Patients were not subjected to any neoadjuvant therapy before surgery. The patient information was obtained from medical charts and follow-up. Informed consent was obtained from each patient. Our study was approved by the Protection of Human Subjects Committee of Zhongnan Hospital.

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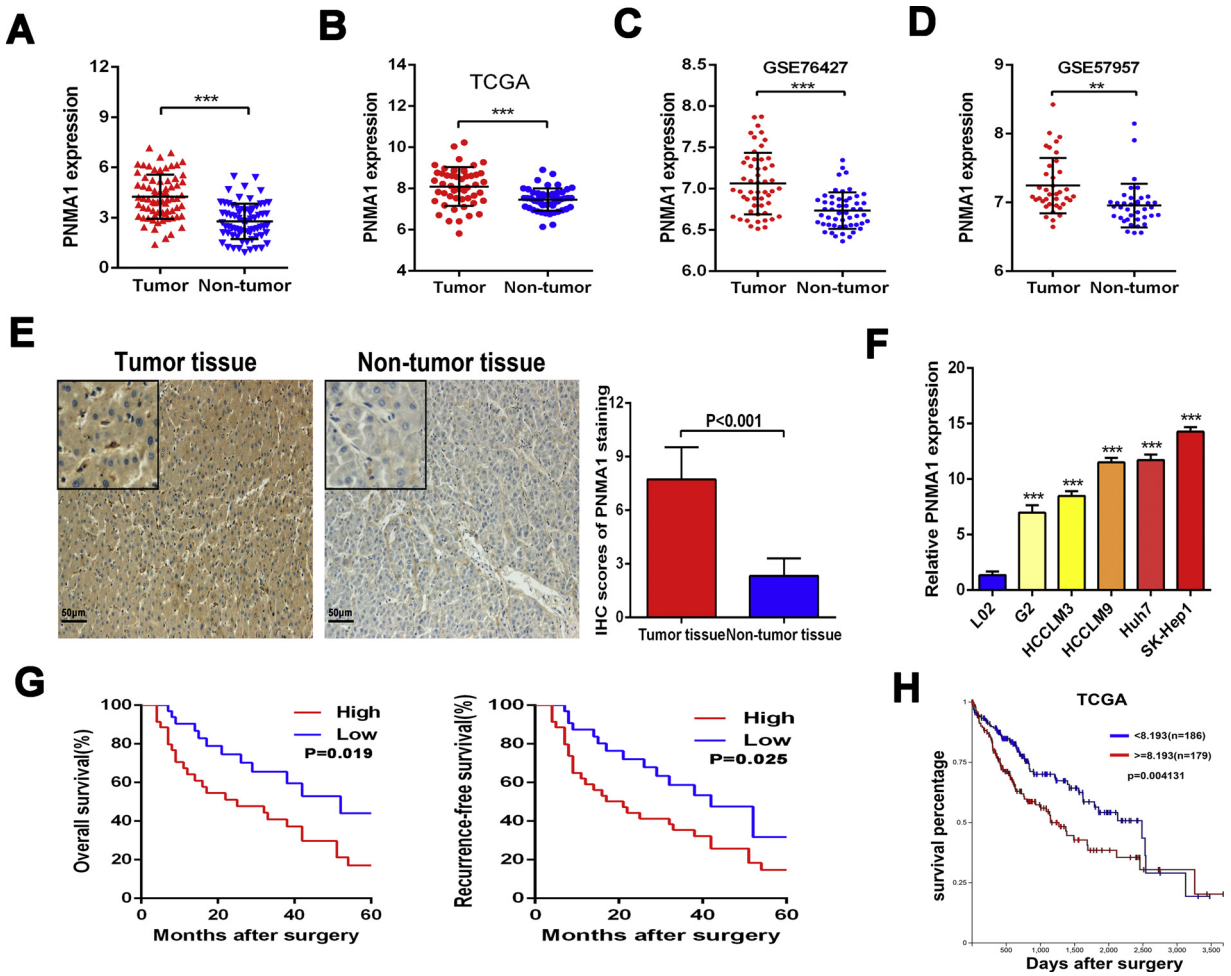

Fig. 1. PNMA1 was overexpressed in HCC and was associated with poor prognosis. (A) PNMA1 mRNA was overexpressed in tumor tissue compared with corresponding adjacent livers in 70 paired HCC patients via quantitative RT-PCR analysis. (B) PNMA1 expression in 50 pairs of HCC samples from TCGA. (C) PNMA1 expression in 52 pairs of HCC samples from GSE76427. (D) PNMA1 expression in 38 pairs of HCC samples from GSE57957. (E) Representative images of immunohistochemistry (IHC) in 15 pairs of HCC tissues showing the protein levels of PNMA1 in HCC and adjacent nontumor tissue. (F) Differential expression of PNMA1 in HCC cell lines (HepG2, HCCLM9, HCCLM3, Huh7 and SK-Hep1) compared with the immortalized normal human hepatic cell L02 cell line. (G) Kaplan-Meier analysis of OS and RFS was compared between patients with high and low expression of PNMA1. (H) Kaplan-Meier analysis of OS in 365 HCC patients from the TCGA dataset. *P < 0.05, **P < 0.01, ***P < 0.001.

2.2. Immunohistochemistry (IHC) and immunofluorescence (IF)

For IHC, paraffin sections were cut to a thickness of 4 μm, the slides were deparaffinized in xylene and rehydrated with ethanol, and the endogenous peroxidase was inactivated with 0.3% hydrogen peroxide. All of the steps were performed using an UltraSensitiveTM S-P kit (Maixinbio, China) according to the manufacturer's protocol. The score of IHC staining was based on the positive percentage and the staining intensity of the stained cells. Staining intensity was graded as follows: 0 (negative), 1 (weakly positive), 2 (moderately positive), and 3 (strongly positive). The percentage of positive cells was scored using five categories (0–4): a score of 0 was given for 0–5%, 1 for 6–25%, 2 for 26–50%, 3 for 51–75 %, and 4 for > 75%. The final staining score was obtained by multiplying the staining intensity and the percentage of positive cells. A staining index score of ≥6 was designated as tumors with high PNMA1expression, whereas a score of ≤5 was designated as low PNMA1 expression. For immunofluorescence, cells were fixed in 4% paraformaldehyde, permeabilized using 0.5% Triton X-100 and incubated with primary antibody and secondary antibodies used according to the manufacturer's protocol. The coverslips were counterstained with DAPI and imaged with a confocal laser-scanning microscope (Olympus FV1000, Tokyo, Japan).

2.3. Cell culture

The human HCC cell lines SK-Hep1, HCCLM9, Huh-7, HCCLM3, HepG2 and the normal liver cell line HL-7702 (L02) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China) and were cultured in high-glucose DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA). All cells were maintained in a humidified incubator at 37 °C with 5% CO2.

2.4. RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted from HCC tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For PNMA1, the reverse transcription was performed using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Tokyo, Japan), which eliminated the interference of genomic DNA. For miR-33a-5p, the reverse transcription PCR was performed using miRNA cDNA Synthesis Kit (ABM Inc., Richmond, BC, Canada). Quantitative real-time PCR was performed using a standard protocol from the SYBR Green PCR kit (Toyobo, Osaka, Japan) in a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA). The GAPDH and U6 level was measured as an internal control. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method. The primers used were as follows: