Loss of PR55α promotes proliferation and metastasis by activating MAPK/AKT signaling in hepatocellular carcinoma

Zhao JiangSheng
Nangjing Medical University
Chen GuoFeng
Nanjing Medical University
Li JingQi
Nanjing Medical University
Liu ShiQi
Nanjing Medical University
Jin Quan
Nanjing Medical University
Zhang ZhengWei
Nangjing Medical University
Qi FUZhen
Nangjing Medical University
Zhang JianHuai
Nangjing Medical University
Xu JianBo (hayyxjb@njmu.edu.cn)
Department of Hepatobiliary Surgery, The Affiliated Huaian NO.1 People's Hospital of Nanjing Medical University, Huai'an, Jiangsu Province, P. R. China. 223001 https://orcid.org/0000-0002-1470-7264

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Abstract

Background: PR55α plays important roles in oncogenesis and progression of numerous malignancies. However, its role in hepatocellular carcinoma (HCC) is unclear.

Methods: PR55α expressions in HCC tissues and paired healthy liver samples were detected using Western blot and tissue microarray immunohistochemistry. We knocked down the expression of PR55α in SMMC-7721 and LM3 cell lines via small interfering and lentivirus. *In vitro* cell counting, colony formation, migration and invasion assays were performed along with *in vivo* xenograft implantation and lung metastases experiments. The potential mechanisms involving target signal pathways were investigated by RNA-sequencing.

Results: PR55α expression level was suppressed in HCC tissues in comparison to healthy liver samples and was indicative of poorer prognosis. Knockdown of PR55α significantly promoted cell proliferation and migration, induced repression of the cell cycle progression and apoptosis *in vitro* while accelerating *in vivo* HCC growth and metastasis. Mechanistic analysis indicated that PR55α silencing was involved with MAPK/AKT signal pathway activation and resulted in increased phosphorylation of both AKT and ERK1/2.

Conclusion: This study identifies PR55α to be a candidate novel therapeutic target in the treatment of HCC.

Background

Global statistics indicate that hepatocellular carcinoma (HCC) represents the seventh most frequently encountered and the third most fatal malignant tumor[1]. Despite improvements in early diagnostic methods and advanced surgical and medical therapy, HCC carries an abysmally poor prognosis, with less than 10% of patients surviving more than 5 years from diagnosis[2]. It is therefore of utmost importance that improved clinical diagnostic and treatment methods are established.

Inactivation of tumor suppressor genes is a critical mechanism of tumorigenesis. For example, TP53INP1 is significantly down-regulated in liver cancer and promotes metastasis[3]. Protein Phosphatase 2A (PP2A) works with protein kinase to maintain the dynamic balance between protein phosphorylation state and dephosphorylation state. Each PP2A consists of one regulatory B subunit, one structural subunit(PP2A-A) and one core catalytic subunit(PP2A-C)[4]. Of these, PP2A-A and PP2A-C are considered to be core enzymes and exist as dimeric complexes, while B regulatory subunits exist independently. The PP2A regulatory B subunits can be classified into PP2A B55/PR55, B56/PR56/PR61, PR48/PR72/PR130 and PR93/PR110. Isomer molecular weights of these subfamily members are indicated by the designated number with these subfamily numbers indicates the molecular weight of the isomer[5].

Recent studies prove that PR55α plays important roles in oncogenesis and progression of numerous malignancies. PP2A/PR55α (PPP2R2A) regulates several crucial pathways that control cell proliferation

and metastasis, including mitogen activated protein kinase (MAPK), c-Myc, YAP, phosphatidylinositol-3-kinase (PI3K)/AKT pathways and the apoptosis machinery[6, 7]. PP2A may function as either tumor suppressors[8, 9] or oncogenes[10, 11]. PR55α has demonstrated to preferentially dephosphorylate phospho-Thr-308 instead of phospho-Ser-473 of the AKT signal pathway in the regulation of cell proliferation and survival of lymphoid cells[12]. In pancreatic ductal carcinoma, elevated PR55α induces cancer cell prolideration via activating many oncogenic signaling pathways, including ERK, AKT, and Wnt[13]. In non-small cell carcinoma of the lungs , PR55α was commonly down-regulated and directly dephosphorylated ATM at S1981, S189 and S367 to promote its presence at double-strand break sites[14].

Our previous study showed that long noncoding RNA GMAN promoted the phosphorylation of eukaryotic translation initiation factor 4B (eIF4B) at serine-422 by preventing the combination of PPP2R2A (PR55α) and eIF4B[15]. EIF4B is a key component of translation initiation and its activity is controlled by MAPK and PI3K pathways[16]. PR55α dephosphorylated eIF4B-Ser422 and repressed mRNA translation and anti-apoptotic protein expression. PR55α is also reported to negatively regulate AKT pathway in HCC[17].

The current investigation uncovers the role of PR55α as a tumor suppressor that is appears to possess a strong correlation with HCC recurrence and poor prognosis. Physiologically, PR55α inhibited cell proliferation and metastasis by repressing the MAPK/AKT pathway.

Methods

Clinical tissues and tissue microarrays

HCC tissues and matched normal samples from The Affiliated Huai'an NO.1 People's Hospital of Nanjing Medical University were used for western blot. Immunohistochemistry (IHC) analysis was performed on tissue microarray kits comprising 80 human HCC tissues and matched 80 healthy tissues that were procured from Outdo Biotech (Shanghai, China). Written informed consent was signed by patients and all experimental protocols were reviewed by the Ethics Committee of Nanjing Medical University.

Cell culture and RNA interference by shRNA

Six HCC cell lines and one normal liver cell line L02 were purchased from KeyGen (Nanjing KeyGen Biotech Co, Ltd, Jiangsu, China). Cells were maintained in 10% fetal bovine serum(FBS)-supplemented Dulbecco's Minimum Essential Medium (DMEM) along with antibiotics in an atmospheric condition of 5% CO2 and at 37□C. Three siRNAs were purchased from Gene Pharma (China). PR55α target sequences utilized in this experiment were the following: GCCUAUGGAUCUAAUGGUUTT for siPR55α#1, GCAGAUGAUUUGCGGAUUATT for siPR55α#2, and GGAAACAUACCAGGUGCAUTT for siPR55α#3. Transfection was performed as previously documented[18].

Western blotting

Western blotting assays were conducted as previously reported[18]. The primary antibodies are as follows PR55α(1/1000, A2185, Abclonal, China), AKT(1/1000, A17909, Abclonal, China), p-AKT-T308(1/1000, AP0304, Abclonal, China), P-AKT-S473 (1/1000, AP0140, Abclonal, China), ERK1/2(1/1000, A4782, Abclonal, China) and p-ERK1/2-T202/Y204(1/1000, AP0472, Abclonal, China).

Immunohistochemistry (IHC)

The slices were deparaffinized in xylol, heated for antigen retrieval with sodium citrate (pH 6.0), and treated by hydrogen peroxide. Then, tissue microarray were incubated with anti-PR55α at 4°C overnight. Finally, secondary antibody and DAB chromogentic agent were added. PR55α staining intensity was graded based on the following scale: 0 (staining not detectable), 1 (faintly yellow, weak staining), 2 (light brown moderately staining), and 3 (brown, strongly staining). A high PR55α expression was marked by colour grades of ≥2. The staining results were assessed by two senior pathologists that remained isolated from the other throughout the experiment.

Cell counting and colony formation assay

Colony formation assays and Cell Counting Kit-8 (CCK-8) assays were used to assess the ability of cell proliferation. 96-well plates were used to house transfected HCC cells at a density of 1×10^3 cells per well. After culturing overnight, the CCK-8 reagent (Dojindo, Shanghai, China) was added to the cells daily at the same time. Two hours later, a microplate reader was used to assess the absorbance values (OD).

Six-well plates were used to contain the transfected cells (1×10^3 cells per well) which was then allowed to undergo a 10-day incubation period. Colonies that obviously contained ≥50 cells were selected for counting. Phosphate buffered saline (PBS) was used to rinse the colonies before they were fixed for 30 minutes with 4% paraformaldehyde and exposed for 2 minutes to crystal violet. Three individual repeats of each experiment were done.

Cell migration and invasion assay

The invasive and migratory abilities of cells were measured by transwell assays. 200ul serum-free medium was used to resuspend 2×10^4 transfected HCC cells before they were placed in the upper cartridge (Milllicell, USA) which was precoated with or without 50ul matrigel. The lower cartridge contained 600ml DMEM medium with 20% FBS. The cells were then allowed to incubate for 24 hours. Paraformaldehyde was then used to fix cells before they were staining using crystal violet. Cells were then photographed and quantified using a microscope for data analysis. The results represented the mean values of three independent experiments.

Cell cycle and apoptosis assay

Cell cycle and apoptosis assays were conducted using flow cytometer (Beckman Coulter). For cell cycle analysis, transfected cells were harvested and fixed overnight in 70% ethanol at 4°C. The cells were then exposed to RNase A before being labeled for 30 minutes with propidium iodide (PI) at 37°C.

Cells were collected and stained with Annexin V-FITC and PI reagents prior to the cell apoptosis analysis. Three separate repetitions were carried out for each experiments.

In vivo tumor assay

BABL/c nude mice were obtained from Nanjing Medical University (Nanjing, Jiangsu, China). Six-week-old female nude mice (n=5) received subcutaneous injections of approximately $2x10^6$ LM3 cells which possessed stable PR55α knockdown or PR55α control to their flanks. The xenograft tumors were dissected and imaged at 4 weeks post-inoculation. Terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labelling (TUNEL) staining and Ki-67 immunofluorescence staining were performed as described previously[18]. For experiments regarding lung metastases, the same cancer cells were administered intravenously into the tail veins of nude mice (n=5). Lung nodules were measured under a dissecting microscope after 40 days of incubation. The Experimental Animal Management Committee of Nanjing Medical University approved all mice experiments which were formulated in strict compliance to established guidelines.

RNA sequencing

RNA sequencing analysis and quantification were utilized to assess changes in mRNA profiles of LM3 cells with PR55α knockdown or PR55α control. Samples were performed in triplicate. The differentially expressed mRNAs were selected with fold change < 0.5 or >2 and possessed a p value < 0.05 by R package edgeR. We also analyzed GO enrichment and KEGG enrichment in the differentially expressed mRNAs.

Statistical analysis

Mean ± standard deviation was used to express all collected data. Analysis was carried out using GraphPadPrism 6. Variances between normal and HCC samples were contrasted using Student's t test. The associations between clinicopathological features and PR55α expression were assessed by Fisher's exact test and chi-square test. Statistical significance was designated as follows: * P<0.05, ** P<0.01 and *** P<0.001. P<0.05 was interpreted as a result that was statistically significant.

Results

PR55α is downregulated in HCC tissues in contrast to healthy specimens

To investigate the potential function of PR55α we assessed the relationship between PR55α and prognosis of HCC. As shown in Figure 1A, lower PR55α expression levels were strongly linked to an overall poorer survival. Likewise, western blotting assays also demonstrated a lower expression of PR55α in HCC samples (Figure 1B). We next evaluated PR55α expression by IHC in a tissue microarray comprising of 80 HCC tissues and adjacent healthy tissues. Our tissue microarray results showed that 66.3% (53/80) HCC tissues had weak or no PR55α expression compared with 17.5% (14/80) cases in

healthy tissues surrounding the tumor (Figure 1C-D). IHC uncovered that PR55α expression in HCC tissues were significantly lower in comparison to healthy samples (P<0.001) (Figure 1E).

In order to explore the potential clinical value of PR55α expression, the clinical characteristics of 80 HCC patients were analyzed. Correlation analysis of clinical parameters showed that PR55α expression correlated inversely with TNM stage and vascular invasion (table 1).

PR55α knockdown induces HCC cell growth and metastasis

In order to fully illustrate the role of PR55α in HCC cell function, Western blotting experiments were done to quantify HCC cell PR55α expression levels. As shown in Figure 2A, PR55α exhibited a higher expression level in SMMC-7721 and LM3 HCC cell lines as compared to L02 normal liver cells. We then used specific siRNA targeted against PR55α in LM3 cells and verified the transfection efficiency by immunoblotting assays and real-time PCR (Figure 2B/C). Stably transfected HCC cell lines were then used for subsequent experiments. Knockdown of PR55α boosted the migration and invasion ability of both SMMC-772 and LM3 using transwell assays compared with control cells (Figure 2D). Moreover, colony formation assays and CCK8 assays indicated that PR55α-shRNA significantly promoted the ability of cell proliferation in SMMC-772 and LM3 in comparison to control cells (Figure 2E/F). When interpreted as a whole, we conclude that the migratory and proliferative ability of HCC cells were attenuated by PR55α.

PR55α knockdown suppresses apoptosis and cell cycle arrest in HCC cells

To verify the inhibitive function of PR55α，a fluorescence-activated cell sorting (FACS) analysis was done to determine the proportion of SMMC-7721 and LM3 cells in each phase of the cell cycle as well as percentage that were apoptosed. We discovered that PR55α knockdown significantly decreased the amount of cells undergoing apoptosis (Figure 3A). As illustrated in Figure 3B, PR55α-shRNA induced a significantly decreased cells in the G0/1 phase while increasing the number of cells in the S phase.

Decrease of PR55α promotes cell growth and metastasis *in vivo*

We next evaluated the inhibitive function of PR55α on liver cancer growth and metastasis *in vivo*. Male nude mice were subjected to subcutaneous injections of LM3 cells that expressed either PR55α-shRNA and PR55α-control stably. Our results showed PR55α-shRNA cells developed into larger tumors than control cells in nude mice (Figure 4A). The weights of tumor xenografts also indicated that PR55α knockdown promoted tumor growth compared with the control cells (Figure 4B). We also tested the xenograft tissues by H&E/ki67 staining and TUNEL assays (Figure 4C). As shown in Figure 4C, shPR55α tumor cells demonstrated a significantly decreased proportion of apoptotic DNA fragments and elevated Ki-67 index compared with the control group. In addition, larger degrees of pulmonary metastasis were found in shPR55α group (Figure 4D). Based on this information, we conclude that the degree of HCC malignancy is inhibited by PR55α both *in vitro* and *in vivo*.

PR55α inhibits HCC progression through MAPK and AKT signaling

To explore the molecular mechanisms of PR55α in HCC, a microarray analysis was performed in LM3 cells with PR55α knockdown and in control cells. The results revealed 166 down-regulated mRNAs and 754 up-regulated mRNAs (Supplementary Table S1). We also uncovered various differentially expressed genes (Figure 5A). KyotoEncyclopedia of Genes and Genomes (KEGG) pathway analysis of the genes that were found to be up-regulated in PR55α silenced cells indicated the potential association between PR55α and MAPK signaling pathway (Figure 5B). Previous investigations have noted the significant involvement of the AKT signaling pathway with PR55α[18]. Sh-PR55α notably increased the amount of phosphorylated AKT-S473, AKT-T308 and ERK1/2-T202/Y204 in SMMC-7721 and LM3 cells, as evidenced by Western blotting (Figure 5C). Together, these results suggested a vital role for PR55α in aberrant MAPK and AKT signaling pathway activation in HCC.

Discussion

PP2A is a serine/threonine phosphatase and the function of PP2A in tumorigenesis is rather controversial, and remains unclear. PR55α (encoded by PPP2R2A) can function as an oncogene or tumour suppressor in different cancers. Previous studies revealed that knockdown of PR55α hindered non-small cell lung cancer cellular growth by increasing JUN T239 phosphorylation[19]. In gastric cancer, PPP2R2A expression is remarkably increased and downregulation of PPP2R2A also inhibits the proliferation and EMT of gastric cancer cells[20]. However, more studies have provided supporting towards the tumor suppressing role of PR55α. Cancer cell invasion and proliferation has been noted to be suppressed by PR55α in diffuse large B-cell lymphoma cells[21] and thyroid cancer[22]. Thus, it is necessary to further investigate the role of PR55α in HCC.

In current research, PR55α is detected frequently at lower levels in HCC tissues, and the negative correlation between PR55α down expression and several malignant characteristics and poor prognosis was confirmed. Functionally, PR55α knockdown significantly induced cell proliferation and invasion, induced cessation of cell cycle progression and facilitated both *in vivo* and *in vitro* cell apoptosis. Furthermore, we demonstrated that sh-PR55α could activate the MAPK/AKT signaling pathway using both microarray analysis and western blot. All these results indicate that PR55α is a tumor suppressor in HCC and may be a reliable biomarker as well as a means to facilitate earlier HCC diagnosis and more effective treatment.

Phosphatases provide both positive and negative regulation for the MAPK pathway at various points. Previous studies showed that PP2A/PR55α facilitated ERK1/2 phosphorylation/activation by activating KSR and Raf[10, 23]. Yet, PR55α has been reported to negatively regulate RAS signaling[24]. PR55α has been reported to inhibit phosphorylation of ERK1/2 in non-small cell lung cancer cells[14] and vascular smooth muscle cells[25]. Microarray analysis showed that knockdown of PR55α significantly induced MAPK signaling. Consistent with microarray analysis, shPR55α promoted Erk1/2 phosphorylation.

In HCC, PI3K/AKT pathway activation represents a significant oncogenic process that has been documented to be commonly activated in HCC[26]. Complete activation of AKT required phosphorylation at

Ser473 by mTORC2 and at Thr308 by PDK1[27, 28]. AKT is activated by various kinases, such as PKA, ACK1 and TNK2, and is inhibited by a variety of phosphatases, such as PP2A, PTEN, PHLPPs and INPP4B[6, 29]. A previous study confirmed that PR55α can directly bind and induce preferential dephosphorylation of phospho-Thr-308 instead of phospho-Ser-473, as evidenced by assays on *in vitro* dephosphorylation using both NIH3T3 and FL5.12 cells[30]. However, PR55α silencing increased baseline phosphorylation of AKT-Thr308 and inhibited Akt-Ser473 phosphorylation upon exposure to insulin-like growth factor-1 in H9c2 cells[31]. More importantly, PR55α significantly regulated AKT phosphorylation at the Thr-308 and Ser-473 residues in pancreatic cancer cells[13] and liver cancer cells[17]. The discrepancy among these previous findings may be due to the differences in cell type or tumor type. Here, our results suggest that decreased PR55α expression is responsible for increased AKT-Ser473 and AKT-Thr308 phosphorylation in HCC.

Conclusion

In conclusion, our results highlight a potential prognostic value of PR55α, and PR55α inhibits proliferation and metastasis of HCC cells likely by inactivating MAPK/AKT signaling.

Declarations

Ethics approval and consent to participate

The present study was approved by the Hospital's Protection of Human Subjects Committee.

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and materials

Related data and materials could be seen in the manuscript and the supplementary files.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

XJB and CGF designed and wrote this manuscript. ZJS, LJQ, LSQ, JQ and ZWG performed experiments. ZJH and QFZ guide the experiment methods. All authors have read and approved final manuscript.

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Tables

Table1 The association between PR55α expression and clinical pathological data in HCC patients.

Features	PR55α level		P
	High expression	Low expression	
Cases	27	53	
Age(years)			
<60	15	33	0.632
≥60	12	20	
Gender			
Man	31	42	0.785
Female	6	11	
Cirrhosis			
Yes	22	40	0.778
No	5	13	
Size(cm)			
<5	7	8	0.364
≥5	20	45	
TNM			
I-II	15	17	**0.043***
III	12	36	
Microvascular invasion			
Yes	6	29	**0.003***
no	21	24	
Histologic grade			
Low	1	5	0.697
Middle	15	26	
High	11	22	

*P<0 .05.

Figures

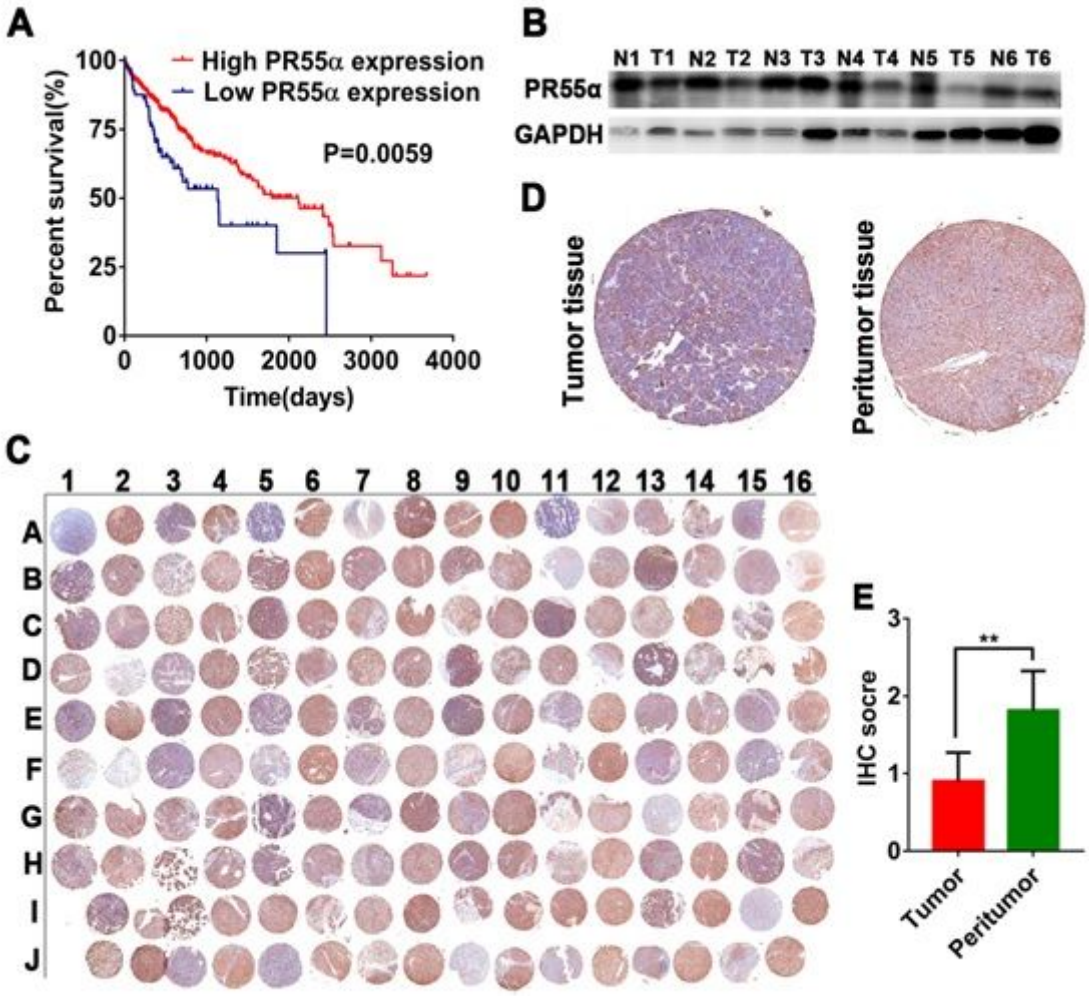

Figure 1

HCC tissues that expressed lower PR55α levels were noted in patients with poorer prognoses. (A) The overall survival of patients with either high (n=82) or low (n=283) PR55α expressions (Data extracted from The Human Protein Atlas) (P=0.0059). (B) PR55α expressions in six paired of HCC tissues and non-adjacent normal liver tissues as determined using Western blotting. (C) Images of immunohistochemical (IHC) staining of PR55α in eighty paired HCC tumor tissue and peritumor tissue. The odd rows were HCC tissues and the even rows were peritumor tissues. (D) Representative IHC-stained images of PR55α in HCC tumor tissue and healthy liver tissues samples. (E) IHC scores of PR55α in HCC and matched healthy liver samples (P <0.01).

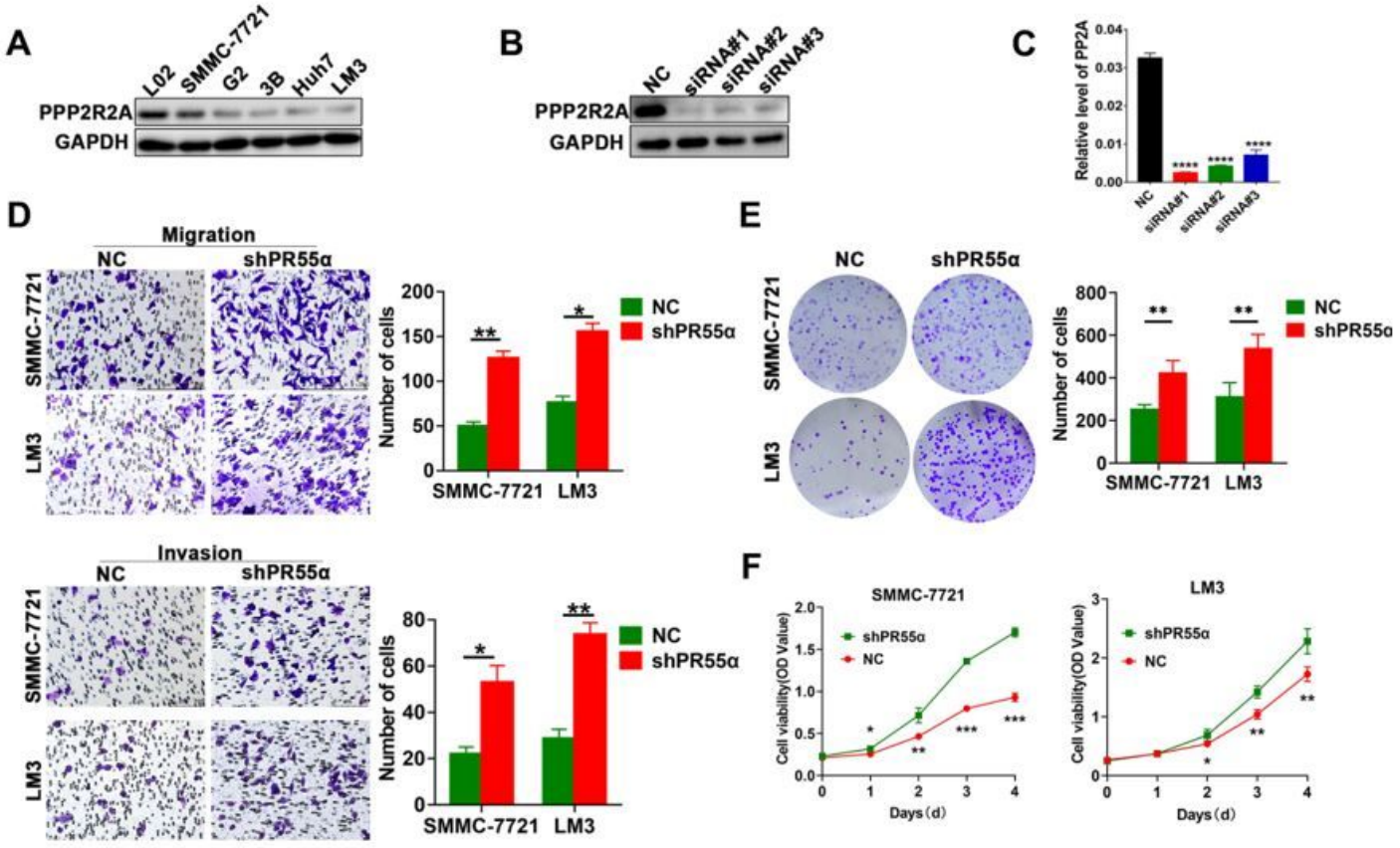

Figure 2

Knockdown of PR55α expression induces cell proliferation and increases cell migration and invasion. (A) PR55α expressions across one normal liver cell line and six lines of liver cancer cells using western blot. siRNA efficacy in knocking down PR55α as tested by western blot (B) and real-time PCR (C). (D) Migration (above) and invasion (down) assays in SMMC-7721 and LM3 lines (200× magnification). (E) Colony formation assays for liver cancer cells transfected with shPR55α. (F) Growth curves in PR55α control and knockdown cells. Each experiment was an average of results obtained from three replicates and is depicted in terms of mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

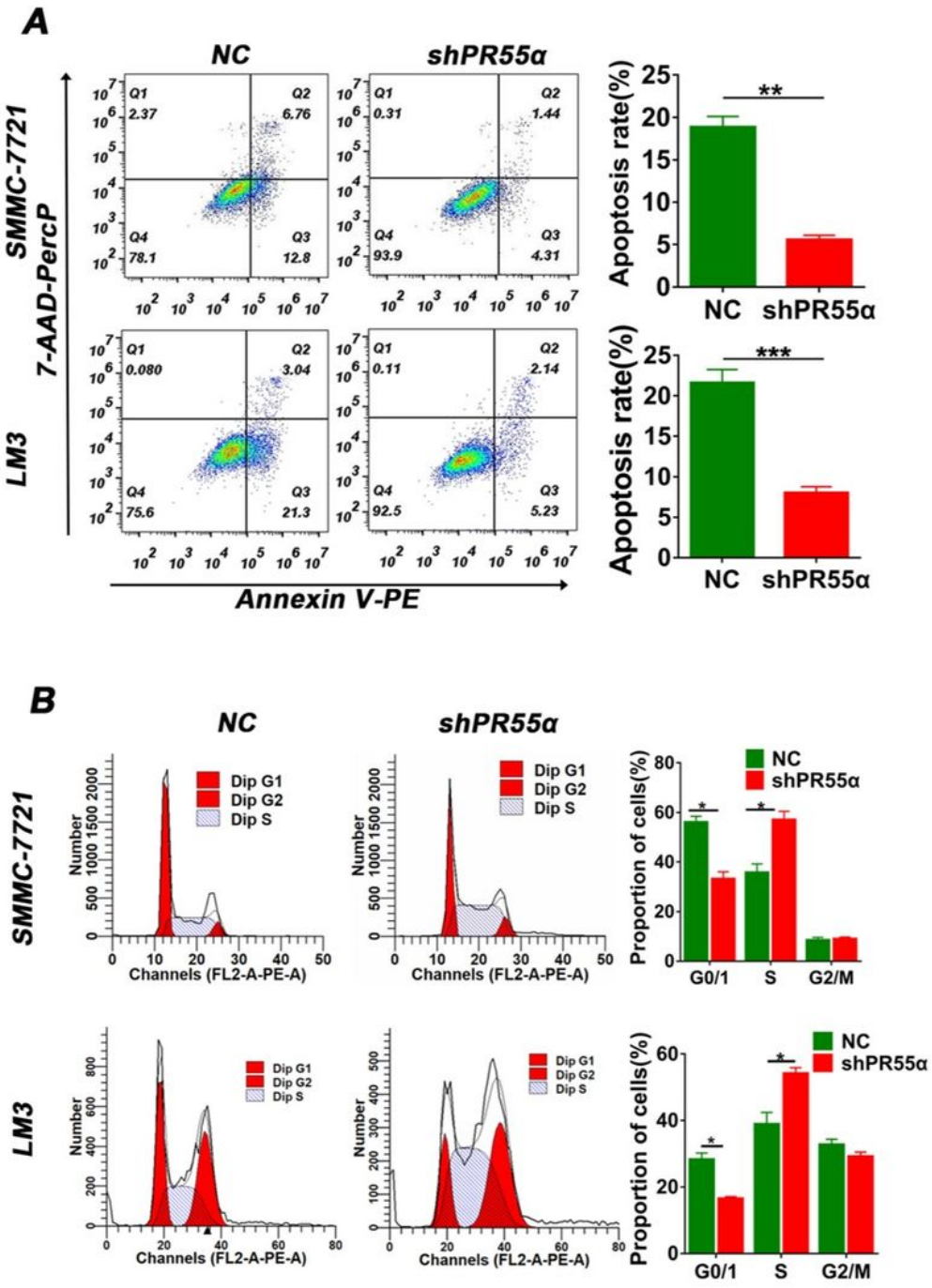

Figure 3

PR55α silencing represses cell cycle arrest and apoptosis. (A) Downrgulation of PR55α expression inhibits apoptosis in SMMC-7721 and LM3 cells in contrast to the NC group. (B) The cell cycles of LM3 and SMMC-7721 cells upon transfection with shPR55α and NC were analyzed by flow cytometer.

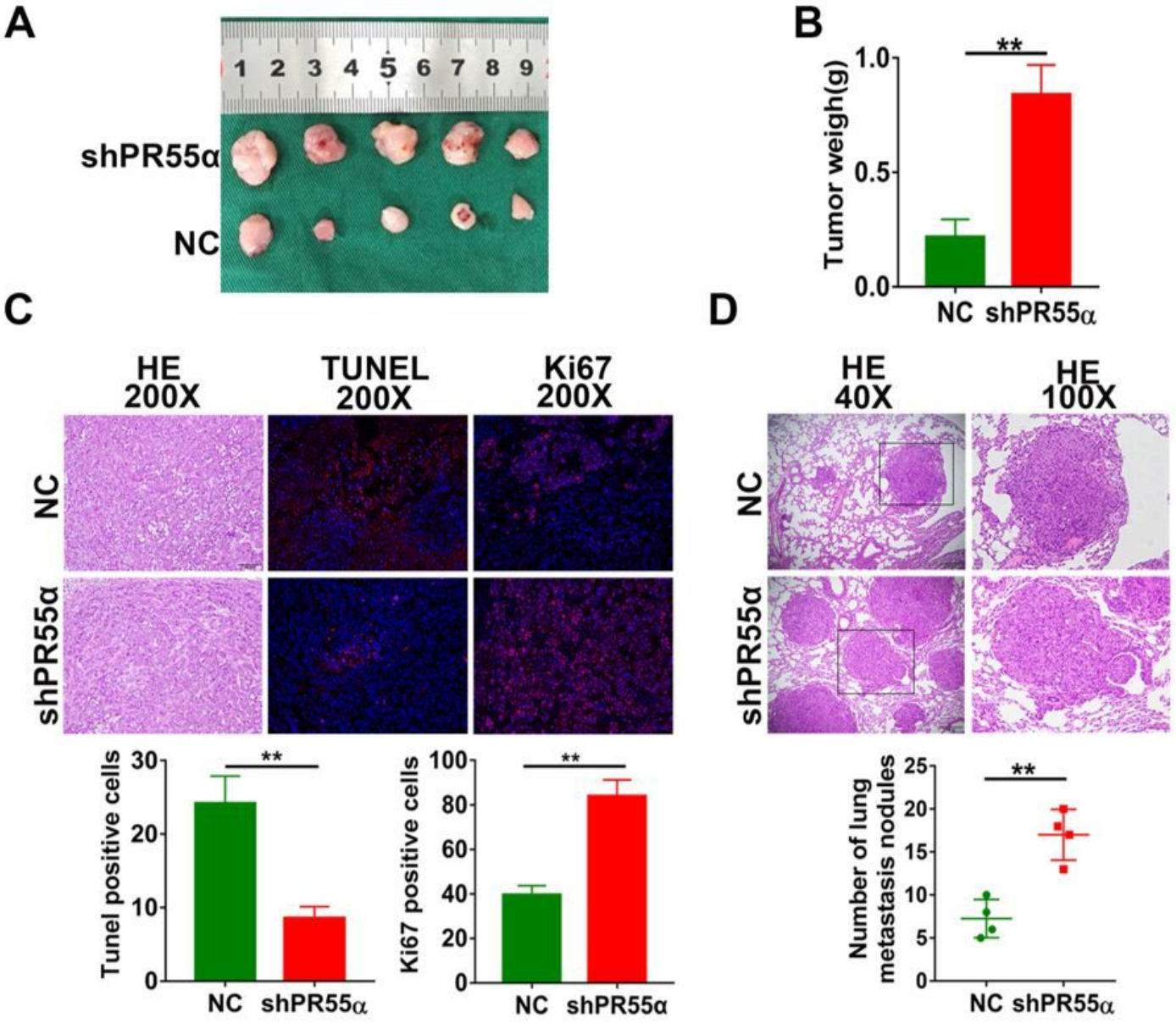

Figure 4

Decrease of PR55α promotes in vivo cell metastasis and growth. (A) Xenograft tumor images in nude mice after 4 weeks of growth (n=5). (B) Analysis of tumor weights in PR55α control and knockdown groups. (C) Representative images of the tumors grown nude mice with HE, TUNEL and Ki-67 (above). Representative images of HE, Ki-67 and TUNEL in tumor xenografts of nude mice (left). Red signal stains was ki67 positive cells and apoptotic cells. Evaluation of TUNEL positive areas (down right) and ki67 expression level (down left) was assessed. (D) Lung metastasis was embedded in paraffin and stained with HE (above). The number of lung metastasis nodules was measured (down) ($P<0.01$).

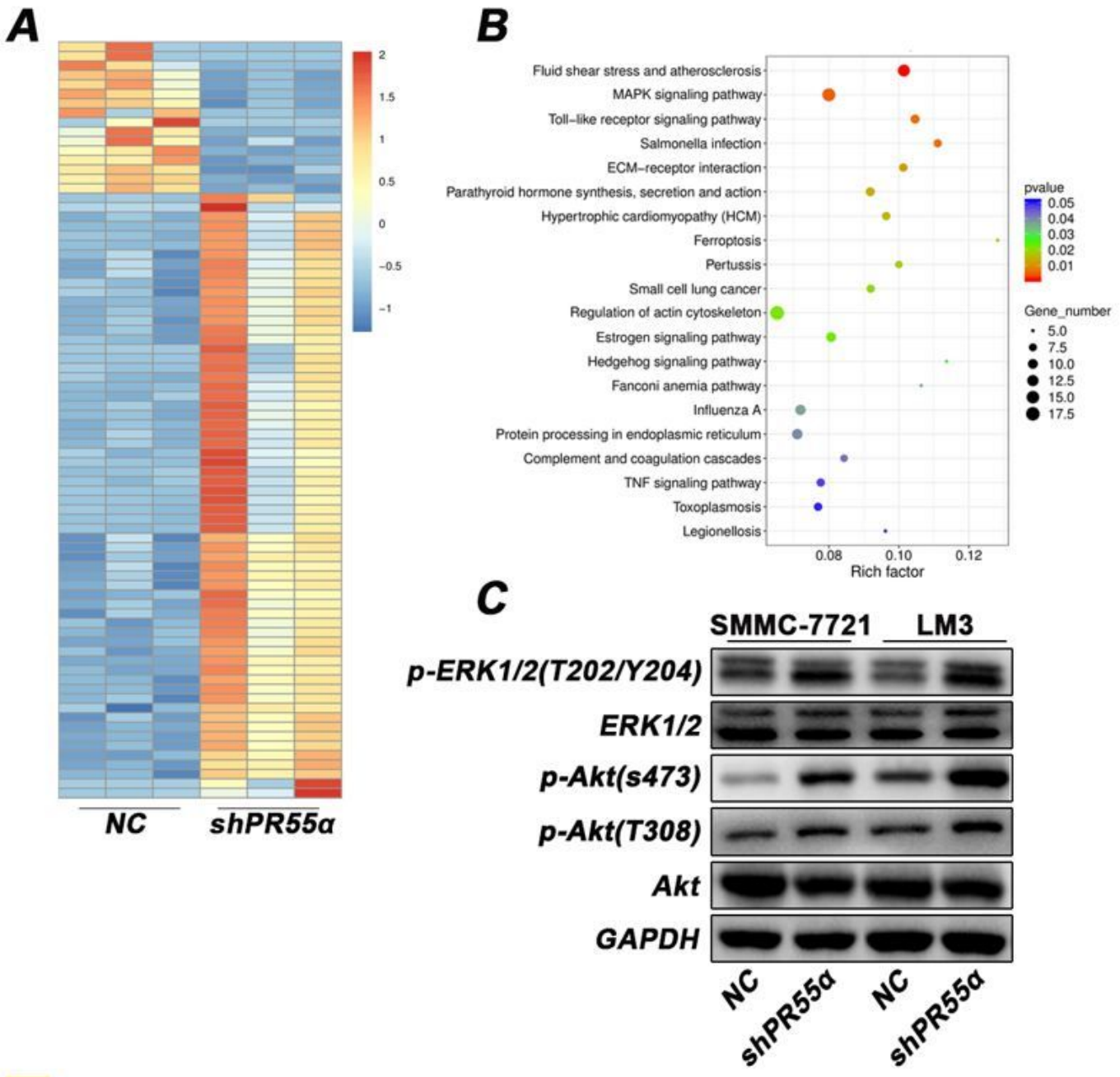

Figure 5

Knockdown of PR55α by shRNA activates AKT and ERK1/2 signaling. (A) Microarray analysis was conducted to screen the mRNAs which can be regulated by PR55α. (B) KEGG pathway analysis revealed the potential signaling pathways involved in PR55α. (C) AKT/ERK signaling pathway protein expressions in HCC cells transfected with shPR55α or shControl as evaluating using western blotting.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTableS1.xlsx