Abstract. Phospholipid scramblase 1 (PLSCR1) serves a function in the pathogenesis and progression of various types of cancer. However, the role of PLSCR1 in human primary liver cancer remains unknown. The aim of the present study was to evaluate the expression of PLSCR1 in primary liver cancer and analyse the clinical significance. In addition, the present study detected and compared the biological behaviours of HepG2 cells with different levels of activated PLSCR1 or silenced PLSCR1. PLSCR1 expression in primary liver cancer tissue samples was examined using immunohistochemistry. Cultured HepG2 cells were infected with lentiviruses to suppress or activate PLSCR1. Reverse transcription-quantitative PCR and western blotting were performed to analyse the effects of silencing or activating PLSCR1 in cell lines at the mRNA and protein levels, respectively. The effects of PLSCR1 expression on cell proliferation, adhesion, migration and invasion were subsequently determined using Cell Counting Kit 8, adhesion, and Transwell migration and invasion assays. PLSCR1 expression in primary liver cancer tissue samples was higher compared with that in adjacent non-cancerous liver tissue samples and normal tissue samples, and positively correlated with the clinical stage. PLSCR1 was effectively downregulated or overexpressed in HepG2 cells using small interfering RNA and lentivirus techniques, respectively. PLSCR1 upregulation promoted cell proliferation, invasion and migration, while PLSCR1 downregulation inhibited these effects. PLSCR1 is highly expressed in primary liver cancer and associated with the clinical stage. Downregulating the expression of PLSCR1 significantly inhibited the proliferation, adhesion, migration and invasion of cancer cells, suggesting that PLSCR1 may be a potential therapeutic target for preventing the progression of primary liver cancer.

Introduction

Primary liver cancer is a common type of cancer worldwide with high morbidity and mortality (1). In China, primary liver cancer is the second leading cause of cancer-related death, and the incidence and mortality rates have continued to rise over the decades (2). Primary liver cancer mainly occurs in the context of long-term chronic liver disease, particularly in hepatitis B and C virus infections and cirrhosis (3). Primary liver cancer is characterized as an asymptomatic disease in early stages and is often detected when at advanced stages. Due to being diagnosed in the advanced stages, primary liver cancer is often incurable (4). With the development of medical science multiple advanced treatments, such as radiofrequency ablation, immunotherapy and molecular-targeted therapy have made significant progress in clinical practice (5). However, new methods for early diagnosis and for therapies are urgently required.

Phospholipid scramblase 1 (PLSCR1) is a Ca²⁺-binding type II endofacial plasma membrane protein that serves an important role in the transbilayer movement of phosphatidylserine and other amino phospholipids (6). These movements are often associated with critical cellular processes, including cell activation, cell proliferation, cell injury responses, tumour suppression, transcriptional regulation and apoptosis (7-12).
The expression of PLSCR1 can be regulated by interferons, epidermal growth factor (EGF) and other cytokines (13). Increasing evidence suggests that PLSCR1 also interacts with several protein kinases that contribute to cell signalling pathways, particularly the EGF receptor (EGFR) signalling pathway (13,14). These studies suggest that PLSCR1 may serve an important role in tumorigenesis. PLSCR1 has been identified to be highly expressed in colorectal cancer and breast carcinoma, suggesting that PLSCR1 is closely correlated with tumour initiation and progression (15-17). However, to the best of our knowledge there are no previous studies on the biological functions and expression levels of PLSCR1 in primary liver cancer.

The present study investigated the expression of PLSCR1 in patients with primary liver cancer and compared biological behaviours among HepG2 cells with different expression levels of PLSCR1. The results of the present study may clarify the association between PLSCR1 and human primary liver cancer.

Materials and methods

Patient information and tissue samples. A total of 44 patients (male=31, female=13; age range, 30-80 years) diagnosed with primary liver cancer at Wuxi No. 2 People's Hospital between January 2010 and December 2014 were included in the present study. The inclusion criteria were as follows: i) Diagnosed to be primary liver cancer treatable with surgical resection; and ii) all the patients had complete information for clinicopathological and prognostic characteristics. Tumour tissue samples and adjacent noncancerous tissue samples were obtained during surgery and normal tissue samples were obtained from 10 normal individuals recruited (male=6, female=4; age range, 45-77 years) from the same hospital within the same time frame. Normal individuals were defined as patients with cholecithiasis requiring surgical treatment and preoperative laboratory tests, their imaging examination did not reveal any hepatic lesions. None of the patients received radiotherapy, chemotherapy or other related anti-tumour therapies prior to surgery. Clinicopathological features, including tumour size, differentiation grade and clinical stage, were collected from pathological reports and checked independently by two observers and finally incorporated in Tables I and II. The present study was approved by the Ethics Committee of Wuxi No. 2 People's Hospital (approval no. 2009-Y-22) and written informed consent was obtained from all the patients prior to surgery and from the normal individuals.

Immunohistochemistry and staining assessment. Immunohistochemical staining was performed on serial 4-µm tissue sections from formalin-fixed (10% formalin for 24 h), paraffin-embedded human primary liver cancer and adjacent normal samples. Dehydration was performed using a descending alcohol series (70, 95 and 100% ethanol). Firstly, paraffin sections were baked in an oven at 60°C for 1 h. The paraffin slides were deparaffinized and washed three times with PBS. The sections were incubated with horseradish peroxidase-labeled anti-rabbit IgG secondary antibody (goat; 1:2,000; cat. no. A-1178, Thermo Fisher Scientific, Inc.) for 30 min at RT. Subsequently, the slides were washed with PBS, incubated with EnVision (Dako; Agilent Technologies, Inc.) for 30 min at RT and then thoroughly washed with PBS. Subsequently, the sections were developed with diaminobenzidine tetrahydrochloride, washed in running water and counterstained with haematoxylin for 2 min at RT. Representative samples were selected and images captured. Finally, the sections were observed under an Olympus light microscope (BX61; Olympus Corporation; magnification, x400). Five random fields were selected and evaluated by two blinded experienced pathologists from Wuxi No. 2 People's Hospital (Wuxi, China). The following definition of immunostaining intensity from Kuo et al (17) was used: Negative, no brown particles in the plasma membrane; positive, light-brown particles, moderately brown particles or deep brown particles in the plasma membrane.

Cell lines and cell culture. HepG2 cell lines (purchased from the Cell Bank of the Chinese Academy of Sciences) were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). All cells were maintained at 37°C in a humidified incubator with 5% CO₂. STR profiling was performed to ensure the cell identity.

Establishment of cell lines with stable PLSCR1 up- or down-regulation. HepG2 cells were seeded in 6-well plates 24 h prior to viral infection at a density of 2x10⁴ cells per well and incubated at 37°C with 5% CO₂ overnight. The cells were transiently transfected with synthesized PLSCR1-specific short hairpin (sh)RNAs (sh-PLSCR1, Applied Biological Materials, Inc.), a PLSCR1-expressing lentivirus (lv-PLSCR1, Applied Biological Materials, Inc.), or the corresponding negative control (shnon or Lvnon, respectively, from Applied Biological Materials, Inc.), or the corresponding negative control (shnon or Lvnon, respectively, from Applied Biological Materials, Inc.) using Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.) after removing the growth medium. Subsequently, 1 µl of polybrene (5 µg/ml) was added to each well. The medium was changed 24 h later and replaced with 2 ml of complete medium, and the incubation was continued. Stable PLSCR1-expressing cell lines were harvested following transfection. Reverse transcription-quantitative (RT-q) PCR and western blotting were performed to investigate the expression levels of PLSCR1. Three days later, well-growing monoclonal strains were selected for frozen storage and subsequent experiments.

RT-qPCR. Transfected cancer cells were processed with TRIzol® (Life Technologies; Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent kit (Takara Bio, Inc.) at 50°C for 45 min. All the experiments were performed following manufacturer's protocols. qPCR was subsequently performed using the SYBR Green I
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Table I. Protein levels of PLSCR1 in primary liver cancer tissue samples, adjacent non-cancerous liver tissue samples and normal liver tissue samples.

| Variable                  | Cases, n | Negative, n (%) | Positive, n (%) | P-value |
|---------------------------|----------|-----------------|-----------------|---------|
| Primary liver cancer      | 44       | 5 (11.36)       | 39 (88.64)      | 0.082a  |
| Adjacent non-cancerous    | 44       | 29 (65.91)      | 15 (34.09)      |         |
| Normal                    | 10       | 9 (90)          | 1 (10)          | <0.001b |

The estimated P-values were adjusted using Bonferroni corrections. *Primary liver cancer vs. Adjacent non-cancerous; †primary liver cancer vs. normal. PSCR1, phospholipid scramblase 1.

Table II. Associations between PLSCR1 expression and clinicopathological features in 44 primary liver cancer patients.

| Variable                  | Cases, n | Negative, n (%) | Positive, n (%) | P-value |
|---------------------------|----------|-----------------|-----------------|---------|
| Age, years                |          |                 |                 |         |
| <60                       | 34       | 3 (8.82)        | 31 (91.18)      | 0.328   |
| ≥60                       | 10       | 2 (20)          | 8 (80)          |         |
| Sex                       |          |                 |                 |         |
| Male                      | 33       | 3 (9.09)        | 30 (90.91)      | 0.411   |
| Female                    | 11       | 2 (18.18)       | 9 (81.82)       |         |
| Tumour size, cm           |          |                 |                 |         |
| ≤3                        | 12       | 2 (16.67)       | 10 (83.33)      | 0.497   |
| >3                        | 32       | 3 (9.38)        | 29 (90.62)      |         |
| Differentiation           |          |                 |                 |         |
| High-moderate             | 41       | 5 (12.20)       | 36 (87.80)      | 0.521   |
| Poor                      | 3        | 0 (0)           | 3 (100)         |         |
| HBs-Ag                    |          |                 |                 |         |
| Positive                  | 35       | 1 (2.86)        | 34 (97.14)      | 0.004   |
| Negative                  | 9        | 4 (44.44)       | 5 (55.56)       |         |
| AFP, µg/l                 |          |                 |                 |         |
| >400                      | 20       | 2 (10)          | 18 (90)         | 0.795   |
| ≤400                      | 24       | 3 (12.5)        | 21 (87.5)       |         |
| Lymphatic metastasis      |          |                 |                 |         |
| Positive                  | 11       | 0 (0)           | 11 (100)        | 0.411   |
| Negative                  | 33       | 5 (15.15)       | 28 (84.85)      |         |
| TNM stage                 |          |                 |                 |         |
| I                         | 3        | 2 (66.67)       | 1 (33.33)       | 0.045   |
| II                        | 19       | 2 (10.53)       | 17 (89.47)      |         |
| III                       | 21       | 1 (4.76)        | 20 (95.24)      |         |
| IV                        | 1        | 0 (0)           | 1 (100)         |         |

PSCR1, phospholipid scramblase 1; AFP, α fetoprotein; TNM, tumour node metastasis; HBs-Ag, hepatitis B surface antigen.
and β-actin reverse, 5'-TACTCCTGCTTGGCTGATCCA-3'. The relative gene expression levels were calculated using the 2-ΔΔCq method (18) and normalized to the internal reference gene β-actin. Each sample was run in triplicate and independently repeated three times.

**Western blotting.** Total protein was extracted from transfected cancer cells using lysis buffer (Beyotime Institute of Biotechnology), and protein concentrations were measured with a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). A total of 30 µl protein extract was separated using 10% SDS-PAGE gels and transferred onto a PVDF membrane. The membranes were blocked with 5% non-fat milk for 60 min at RT, followed by incubation with primary antibodies against PLSCR1 (1:500; cat. no. ab250216; Abcam), EGFR (1:500; cat. no. ab52894; Abcam), and β-actin (1:500; cat. no. ab8227; Abcam) overnight at 4˚C, then treated with a Horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:1,000; cat. no. ab150077; Abcam) for 2 h at RT. The protein bands were visualized using an enhanced chemiluminescence system (Beyotime Institute of Biotechnology). β-actin was used as a loading control for normalization and images were analyzed using ImageJ software version 1.52 (National Institutes of Health).

**CCK8 assay.** Transfected cancer cells (8x10^4 per well) were seeded in 96-well plates in quintuplicate and incubated at 37˚C. DMEM was removed 24 h later and replaced with 100 µl of fresh medium. A Cell Counting Kit-8 (CCK8; Dojindo Molecular Technologies Inc.) assay was used to evaluate cell proliferation according to the manufacturer's instructions. A CCK8 solution (10 µl) was added to each well, and the cells were incubated for 4 h at 37˚C in a CO₂ incubator. The absorbance at 450 nm was determined by an ELISA plate reader.

**Wound heal assay.** Cells (3x10^5/well) were seeded and grown in 6-well plates until confluent (90%) cell monolayers were formed. Then, a sterile 10-µl pipette tip was used to make a uniform scratch along the bottom of each well. The horizontal distance between the sides of the scratch was measured. The cells were washed with PBS three times to remove detached cells and incubated with serum-free medium at 37˚C for 24 h after replacement of the culture medium. Then, wound size was observed and images were captured under an inverted fluorescence microscope (Nikon Corporation; magnification: x20).

**Transwell migration and invasion assays.** Transwell chambers were used for transfected cancer cell migration and invasion assays. The 24-well culture inserts (BD Biosciences) with 8-µm pores coated with Matrigel (1 mg/ml; BD Biosciences) were used. The membranes of upper chamber were coated with Matrigel (BD) and then incubated for 6 h at 37˚C. For the migration assay, 2x10^5 transfected cells suspended in 200 µl of serum-free medium were seeded in the upper chambers. Medium containing 20% FBS was added to the lower chamber. For the invasion assay, a Chemicon Cell Invasion Assay kit (Chemicon International; Thermo Fisher Scientific, Inc.) was used. The non-migrating/invading cells were removed from the upper surface of the membrane with cotton swabs following an incubation for 24 h at 37˚C, and the migrating/invading cells were stained with 0.1% crystal violet for 30 min at RT and images captured with an inverted fluorescence microscope (magnification: x20; Nikon Corporation).

**Adhesion assays.** A 96-well plate was coated with laminin (Ln, 20 µg/well) or fibronectin (Fn, 10 µg/well) for 2 h at 37˚C, washed with PBS twice and sealed with 1% BSA (Thermo Fisher Scientific, Inc.). A single-cell suspension (3x10^5 cancer cells/ml) was prepared with transfected cells and serum-free medium. Then, 200 µl of suspension was added to the coated plates. The cells were incubated at 37˚C with 5% CO₂ for 2 h and then the non-adherent cells were gently washed off. Each well was fixed with 4% formaldehyde for 10 min at RT and stained with 0.5% hexamethylenetetramine for 20 min at RT. Subsequently, 100 µl of 10% SDS was added to each well. An ELISA plate reader was used to assess the absorbance at 570 nm.

**Statistical analysis.** SPSS 18.0 software (SPSS, Inc.) was used to perform all statistical analyses. Continuous variables are presented as the mean ± SD and comparisons between groups were performed using one-way ANOVAs followed by post-hoc Tukey's tests. Categorical data are expressed as a proportion (%) and were compared using the χ² test, McNemar's test or Fisher's exact test. Bonferroni's corrections were used to reduce type I errors as the primary liver cancer data in Table I had been analysed more than once. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Expression of PLSCR1 in primary liver cancer tissue samples and the associations of PLSCR1 levels with clinicopathological variables.** Fig. 1 and Table I show that PLSCR1 protein was detected in liver tissue samples through immunohistochemical staining. The PLSCR1 protein was mainly located at the cancer cell membrane (Fig. 1). The expression of PLSCR1 in the primary liver cancer sample group was significantly higher compared with the normal group, while no significant difference was observed between primary liver cancer tissue samples and the adjacent non-cancerous liver tissue samples (Fig. 1 and Table I). As shown in Table II, PLSCR1 expression was observed in almost all primary liver cancer patients (88.64%, 39/44 patients), whereas the positive rate of PLSCR1 expression in the normal group was only 10%. The positive rate of PLSCR1 expression increased with the increase of tumour size, although the difference was not statistically significant (83.33% vs. 90.62%). The expression of PLSCR1 in primary liver cancer was correlated with the hepatitis B virus (HBV) infection status. Furthermore, it was identified that the positive rate of PLSCR1 expression in TNM stage III/IV samples was significantly higher compared with that in stage I/II samples, suggesting that the expression level of PLSCR1 is correlated with clinical stage. However, no correlations were identified between PLSCR1 expression and other clinicopathological characteristics, including patient sex, tumour size, tumour differentiation, α fetoprotein level and lymph node metastasis status.
PLSCR1 expression in transfected cancer cells. Following transfection with sh-PLSCR1, Lv-PLSCR1 or the corresponding negative control, the expression levels of PLSCR1 in the various groups was assessed using RT-qPCR and western blotting. The sh-PLSCR1 cell lines expressed the lowest levels of PLSCR1 at both the mRNA and protein levels, while significantly increased expression levels of PLSCR1 was observed in the Lv-PLSCR1 group compared with the other three groups (Fig. 2).

Effects of PLSCR1 expression on cell proliferation, adhesion, migration and invasion. Following transfection, PLSCR1 was overexpressed in the Lv-PLSCR1 cells, and the expression levels of PLSCR1 were reduced in the sh-PLSCR1 cells as aforementioned. To investigate the effects of PLSCR1 on the biological behaviour of HepG2 cells, a series of assays including CCK8, adhesion and Transwell migration and invasion assays were performed. As shown in Fig. 3A and B, the CCK8 assays indicated that compared with the Lv-PLSCR1 cells, the sh-PLSCR1 cells exhibited significantly inhibited proliferation at 24 and 48 h. Representative micrographs of Transwell filters are shown in Fig. 3C. Transwell migration and wound heal assays demonstrated that PLSCR1 overexpression resulted in an increased number of migrating Lv-PLSCR1 cells and promoted the migration of cancer cells (Fig. 3C-E). In addition, the number of invasive HepG2 cells was significantly decreased in the sh-PLSCR1 group compared with the Lv-PLSCR1 group (Fig. 4A and B). Ln and Fn adhesion assays were performed to assess HepG2 cell adhesion following transfection. These assays demonstrated that HepG2 cell adhesion was inhibited in the sh-PLSCR1 group.
at 48 h (Fig. 4C and D). Collectively, the data suggested that PLSCR1 upregulation may promote HepG2 cell proliferation, migration and invasion and that PLSCR1 downregulation can inhibit these effects.

**Suppression of PLSCR1 expression blocks the EGFR signalling pathway.** As shown in Fig. 5, expression of EGFR was decreased in cancer cells following PLSCR1 inhibition but increased in the PLSCR1-overexpression cells.

**Discussion**

Primary liver cancer is considered a serious health problem of global importance (19). The majority of patients are asymptomatic in the early stage but are often diagnosed in advanced stages, which are resistant to therapies (4,20). Surgery is an optimal approach for primary liver cancer treatment, but the majority of patients may not be able to benefit from surgery due to a high rate of recurrence (21). Therefore, early diagnosis and the identification of new therapeutic targets will benefit patients with primary liver cancer.

As aforementioned, increasing evidence suggests that PLSCR1 serves important roles in cell proliferation, differentiation, apoptosis and cancer pathogenesis and progression (17). Studies have demonstrated that PLSCR1 overexpression is associated with the differentiation of human myeloid leukaemia cells into granulocytes and inhibits the growth of ovarian cancer cells (9,22). Other studies have revealed that suppression of PLSCR1 inhibits the growth and metastasis of colorectal cancer (16,23). Nevertheless, the roles of PLSCR1...
in the pathogenesis and progression of primary liver cancer remain to be elucidated.

In the present study, there was a strong correlation between PLSCR1 expression and tumor stage. Univariate analysis with the Cox regression model demonstrates that increased PLSCR1 expression indicates a poor prognosis for patients with colorectal cancer (16,17). Based on these results and previous studies, it was hypothesized that PLSCR1 may serve

Figure 4. Lentivirus-mediated overexpression of PLSCR1 promotes HepG2 cell invasion and adhesion in vitro. (A) Representative images of cell invasion determined by Transwell invasion assays of cells transfected with (a) shnon, (b) sh-PLSCR1, (c) Lvnon or (d) Lv-PLSCR1. (B) Quantification of the Transwell invasion assays. (C) Ln and (D) Fn adhesion assays. Data are presented as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001. OD, optical density; PSCR1, phospholipid scramblase 1; sh, short hairpin RNA; lv, lentivirus; Ln, laminin; Fn, fibronectin.
a vital role behind the tumorigenesis of primary liver cancer. The present study also demonstrated that the expression of PLSCR1 in primary liver cancer was associated with the HBs-Ag status. Yuan et al (24) reported an important role for PLSCR1 in the host defence against HBV infection and the development of primary liver cancer in patients with HBV, which are consistent with the findings of the present study.

To evaluate the biological function of PLSCR1, siRNA and lentiviral technologies were used to regulate the expression of PLSCR1 and the changes in expression were verified by RT-qPCR and western blotting. The results demonstrated that proliferation, adhesion, migration and invasion were blocked following the suppression of PLSCR1 expression and promoted following upregulation of PLSCR1 expression, suggesting that PLSCR1 could be a potential therapeutic target for hepatocellular carcinoma. Previous studies have indicated that the EGFR signalling pathway is inhibited in cells with knocked down PLSCR1 expression (9,22). Stimulation of EGFR-expressing cells via EGF causes PLSCR1 to interact with activated EGFR and SHC Adaptor Protein 1 (Shc), which results in the tyrosine phosphorylation of PLSCR1 (25). PLSCR1 is a substrate of multiple kinases, involved in cell proliferation, differentiation or apoptosis including tyrosine-protein kinase ABL1, proto-oncogene tyrosine-protein kinase Src (Src) and protein kinase (14,26-28). In EGF-treated cells, activated Src kinase causes the tyrosine phosphorylation of PLSCR1, which is required for the interaction of PLSCR1 with Shc in response to EGF stimulation (14). In the present study, the production of EGFR was decreased following suppression of PLSCR1 expression and increased following upregulation of PLSCR1. Therefore, suppression of PLSCR1 expression may block the EGFR signalling pathway and inhibit the proliferation, adhesion, migration and invasion of HepG2 cells. However, a limitation of the present study is that the downstream proteins inside the EGFR signal penetration pathway were not measured, which would further support the reduction in EGFR signalling. Lack of in vivo experiments is another limitation of the present study and which should be further investigated.

In summary, the present study indicated that PLSCR1 was highly expressed in primary liver cancer and associated with the tumour stage. Downregulating the expression of PLSCR1 significantly inhibited the proliferation, adhesion, migration and invasion of cancer cells. PLSCR1 overexpression may serve important roles in tumorigenesis and tumour progression, and suppressing PLSCR1 expression may be beneficial for the treatment of primary liver cancer. However, further research needs to be conducted to explore the potential molecular mechanisms of the signalling pathways involved.

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Availability of data and materials
The datasets used and analysed during the current study are available from the corresponding author on reasonable request.
Authors' contributions

LG, YZ and QX designed the study, performed the experiments and analysed the data statistically. LG wrote the manuscript. JH, PH and GW performed data collection. LZ and JL made substantial contributions to the conception of this study. JN and HT conducted data processing and statistical analyses, and made critical revisions. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Wuxi No. 2 People's Hospital, and written informed consent was obtained from all the patients prior to surgery.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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