

# Proteomic identification of RhoA as a potential biomarker for proliferation and metastasis in hepatocellular carcinoma

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**Abstract** Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world, and there is an urgent need to discover novel factors that can act as biomarkers for prognostic assessment and therapeutic targets of HCC. In this study, highly purified plasma membrane proteins from clinical tissue samples were obtained using a strategy combining sucrose density gradient centrifugation and subsequent phase partition. Using a two-dimensional gel electrophoresis and MALDI-Q-TOF MS/MS-based proteomics approach, we identified 13 plasma membrane-associated proteins that were differentially expressed in HCC and normal liver tissues. Of those, RhoA was one of the most significantly upregulated proteins in HCC, and its overexpression was confirmed using Western blotting. Immunohistochemistry suggested a link between RhoA expression and poor differentiation and clinicopathologic stage. Suppression of RhoA expression in HepG2 and Hep3B cells by RNA interference led to significant inhibition of cell growth, induction of apoptosis, and a decrease in migration. Our data suggest that RhoA may serve as a potential biomarker and an attractive therapeutic target for HCC.

**Keywords** RhoA · Hepatocellular carcinoma · Biomarker · Proteomics

## Introduction

Primary liver cancer, also known as hepatocellular carcinoma (HCC), is the fifth most common cancer, with approximately one million new diagnoses annually, and it is one of the most deadly cancers, with approximately 600,000 yearly deaths attributed to this disease [1, 2]. HCC accounts for 80–90% of all cases of liver cancer, and its prevalence is highest in China, Southeast Asia, and sub-Saharan Africa [3, 4]. HCC begins in the main cell type in the liver and most frequently occurs in those people with liver disease and scarring called cirrhosis [5, 6]. As a precancerous lesion, cirrhosis typically occurs in patients who suffer from chronic hepatitis B or C infection or who have a long history of alcohol abuse [7, 8].

Proteomics is a promising approach that may overcome some of the limitations of previous approaches for the elucidation of the molecular mechanisms underlying many biological processes [9, 10]. Analysis of proteomic data has led to the identification of a number of novel, noteworthy biomarkers and signaling pathways [11, 12]. In recent decades, proteomic approaches have been successfully used in the investigation of many types of cancer, including breast, lung, colon, and stomach carcinomas [13, 14].

Nevertheless, few proteomic studies focusing on subcellular fractions such as the plasma membrane have been performed as yet because of the difficulty of obtaining purified plasma membrane proteins [15, 16]. To date, there are very few proteomic reports concerning the plasma membrane of HCC cells, especially at the level of clinical tissue samples from patients with HCC. Some comparative proteomic studies investigating HCC and normal cell lines

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or tissues have revealed significant and reproducible changes in the expression level of a number of proteins including metabolic enzymes, signal transduction factors, and oncoproteins; however, few of these proteins were found to change their expression levels in concert, reflecting regional variability or tissue heterogeneity [17, 18]. Also, few of these proteins have been subjected to further functional analysis concerning their roles in HCC carcinogenesis, and therefore we lack the complete understanding of the underlying biological processes that is required for clinical applications.

In this study, a strategy combining sucrose density gradient centrifugation and subsequent phase partition was applied to obtain plasma membrane proteins of high purity. The plasma membrane-associated proteins that were differentially expressed in cancer and in the corresponding normal tissues were profiled. One of these proteins, RhoA, was chosen for further clinical validation and functional investigation. The data resulting from this work are expected to bring about an improved understanding of the potential biomarker RhoA in the initiation and progression of HCC and thereby facilitate the translation of experimental findings into clinical applications.

## Materials and methods

### Clinical specimens

All tissue specimens were collected at the West China Hospital of Sichuan University. For two-dimensional electrophoresis (2-DE), fresh HCC and pair-matched adjacent normal tissues were collected from 12 HCC patients who underwent surgical resection. For immunohistochemical (IHC) analyses, paraffin-embedded HCC specimens were collected from 72 patients who underwent surgical resection. A summary of the clinical and pathologic profiles for these patients is shown in Table 1. The project was approved by the institutional ethics committee of Sichuan University. Informed consent for research was received from all patients prior to analysis.

### Preparation of plasma membrane-associated proteins

The tissues were dissolved in homogenization buffer (50 mM HEPES, pH 7.4, 1 mM CaCl<sub>2</sub>, 1 mM EDTA, 1 mM vanadate, and 1 mM phenylmethylsulfonyl fluoride) and subjected to sucrose density gradient centrifugation (40%, 35%, 30%, 25%, 20%, 15%, 10%, and 5%) as previously described [19]. Solutions containing 40% (w/w) polyethylene glycol 3350 and 20% (w/w) Dextran T-500 were freshly prepared for subsequent phase partition [20]. The samples were subjected to Western blotting for evaluation of purification.

**Table 1** Clinicopathological correlation of RhoA with HCC parameters

Clinical features	Number (%)	Staining score	P value
Gender			
Male	58 (80.6)	5.8±2.3	0.72
Female	14 (19.4)	5.4±2.2	
Age (years)			
≤30	6 (8.3)	5.7±2.3	0.17
31–59	35 (48.6)	5.5±2.2	
≥60	31 (43.1)	5.3±2.2	
Differentiation			
Good	21 (29.2)	4.6±2.2	0.03
Moderate	29 (40.3)	5.7±2.3	
Poor	22 (30.5)	6.1±1.9	
T stage			
T <sub>1</sub>	17 (23.6)	5.2±2.7	0.80
T <sub>2</sub>	29 (40.3)	5.9±2.4	
T <sub>3</sub>	14 (19.4)	5.4±2.3	
T <sub>4</sub>	12 (16.7)	5.8±2.6	
N stage			
N <sub>0</sub>	28 (38.9)	4.4±1.8	0.04
N <sub>1</sub>	44 (61.1)	6.8±2.0	
M stage			
M <sub>0</sub>	59 (81.9)	4.1±1.4	<0.01
M <sub>1</sub>	13 (18.1)	7.3±2.2	
Clinical stage			
I–II	40 (55.6)	4.3±1.8	<0.01
III–IV	32 (44.4)	6.9±2.1	
Alcohol use			
No	53(73.6)	5.3±2.0	0.79
Yes	19(26.4)	5.9±2.6	
Smoking			
No	45(62.5)	5.4±2.1	0.91
Yes	27(37.5)	5.7±2.4	

### Proteomics

Approximately 300 µl of sample containing 1 mg of protein was applied to standard 2-DE gels [21]. The gels were stained in Coomassie Brilliant Blue R-250 (Bio-Rad) and then analyzed using PDQuest-7.1 software (Bio-Rad). The spots that showed a greater than 3.0-fold difference between conditions were chosen for in-gel digestion using Trypsin Gold (Promega) according to the manufacturer's instructions. MALDI-Q-TOF mass spectrometry was performed on a Q-ToF Premier mass spectrometer (Waters). The mass spectrometry data were acquired as peak list (PKL) files using MassLynx Version 4.0 software (Waters) and subsequently processed using the public MASCOT program (Matrix Science) [22].

### Immunohistochemistry

The sections were incubated with a mouse monoclonal antibody against RhoA (ab54835, Abcam) and stained with Envision System horseradish peroxidase (DakoCytomation, Inc.) according to the manufacturer's instructions. For statistical analysis, total staining of RhoA was scored as the product of the staining intensity (on a scale of 0–3: negative 0, mild 1, moderate 2, and strong 3) × the percentage of positive cells (recorded on an ordered categorical scale: 0, zero; 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, 76–100%), resulting in a scale of 0–12 [23].

### Cell culture

The human HCC cell lines HepG2 and Hep3B were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

### RNA interference

Four pairs of RhoA-specific small interfering RNA (siRNA), a scrambled siRNA oligonucleotide (used as a negative control; siNC), and a siRNA oligonucleotide targeting glyceraldehyde-3-phosphate dehydrogenase (used as a positive control) were synthesized by Ambion, Inc. The siRNA transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. A pilot experiment was carried out to identify two RhoA-specific siRNAs (siRhoA-1 and siRhoA-2) that were shown to inhibit RhoA expression by Western blotting.

### Western blotting

The protein extracts were subjected to 12% SDS-PAGE, transferred to PVDF membranes (Millipore), and incubated with primary antibodies against  $\alpha$ -Na,K-ATPase (ab2871, Abcam), mitochondrial complex IV subunit I (ab14705, Abcam), calnexin (ab2798, Abcam), and RhoA (ab54835, Abcam). The blots were probed with secondary antibodies conjugated with horseradish peroxidase and visualized using an enhanced chemiluminescence system (Pierce).

### MTT and colony formation assay

MTT assay was performed using an MTT reagent (Roche). The cell vitality index was calculated using the following formula: vitality index = OD treated wells / OD control wells × 100. For colony formation assay, cells were seeded in six-well plates at  $3 \times 10^2$  cells per well and incubated

overnight. After transfection of siRNA, the cells were allowed to grow continuously for an additional 10 days. The cells were fixed with methanol and stained with crystal violet (Sigma).

### Flow cytometry, TUNEL, and Hoechst staining

Cells were seeded in six-well plates at  $2 \times 10^5$  cells per well and harvested at 72 h post-transfection. After washing with PBS, the cells were then resuspended and incubated in a propidium iodide/Annexin V solution (R&D Systems). Flow cytometry was carried out on a FACS Aria flow cytometer (BD Biosciences). TUNEL and Hoechst staining were performed according to the instructions of the DeadEnd<sup>TM</sup> Fluorometric TUNEL System (Promega) and Hoechst 33258 (Sigma), respectively.

### Cell migration and wound healing assays

Cell migration assay was performed on 8.0- $\mu$ m pore, 24-well transwell plates (Millipore). Cells were seeded into the upper chamber, which contained serum-free medium, while the lower chamber was filled with complete medium that acted as a chemoattractant. Cells attached to the lower side were fixed in 4% paraformaldehyde in PBS for 20 min and stained for 10 min with 0.5% crystal violet. The number of migrated cells on the lower side of the membrane was counted in five microscopic fields. For wound healing assay, cells were seeded in six-well plates and cultured overnight. Adherent cells were scratched with a 100- $\mu$ l micropipette tip and monitored for migration into the wound [24].

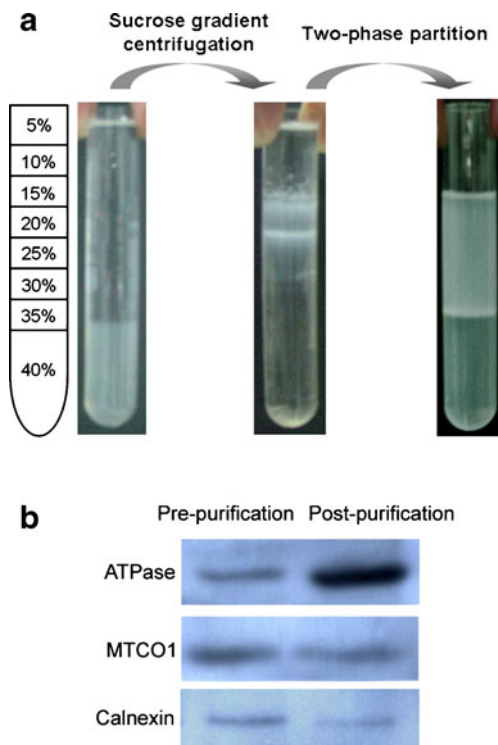
### Statistical analysis

All quantitative data were recorded as means  $\pm$  SD. The one-way analysis of variance was used to analyze differences among multiple groups. Statistical significance was defined as  $P < 0.05$  for all analyses. Computations were performed using the statistical package SPSS 11.5 (SPSS).

## Results

### Preparation of plasma membrane-associated proteins

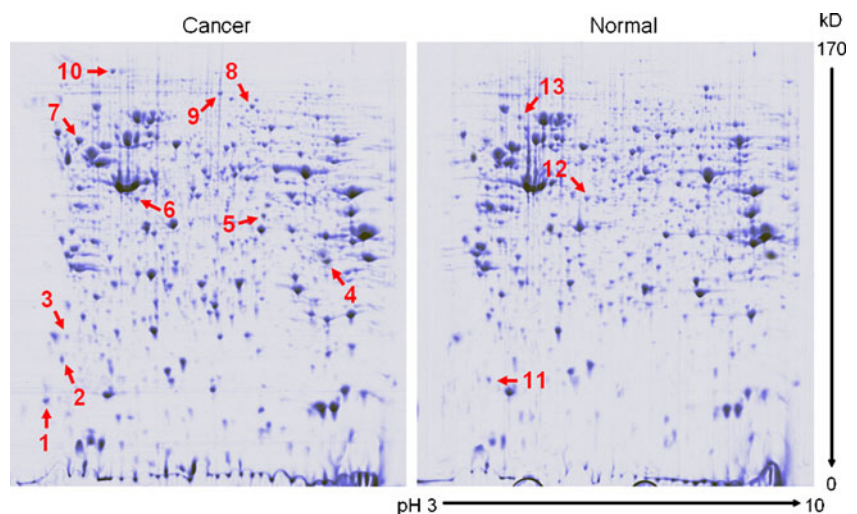
In this study, a strategy combining sucrose density gradient centrifugation and subsequent phase partition was used to obtain plasma membrane proteins of high purity (Fig. 1a). Western blotting showed that the level of  $\alpha$ -Na,K-ATPase (a plasma membrane marker) was significantly enriched after our purification strategy, while the levels of mitochondrial complex IV subunit I (MTCO1, a mitochondrial



**Fig. 1** Strategy combining sucrose density gradient centrifugation and subsequent phase partition to obtain plasma membrane-associated proteins. **a** The post-nuclear fractions were subjected to sucrose density gradient centrifugation (40%, 35%, 30%, 25%, 20%, 15%, 10%, and 5%). The crude membrane fractions were collected and mixed with a solution of 40% (*w/w*) polyethylene glycol 3350 and 20% (*w/w*) Dextran T-500 for further phase partition at 4°C. **b** Western blotting (25 µg protein per lane) showed that the level of ATPase was greatly increased after purification, indicating that plasma membrane proteins were enriched

membrane marker) and calnexin (an endoplasmic reticulum membrane marker) were reduced (Fig. 1b). These results demonstrate that the plasma membrane, but not the

**Fig. 2** Profile of plasma membrane-associated proteins that were differentially expressed in HCC and normal liver tissues. Averages of 758 and 713 spots were visualized in 2-DE gels for HCC and normal liver tissues, respectively. The identified differentially expressed plasma membrane-associated proteins are indicated with arrows



mitochondrial or endoplasmic reticulum membranes, was greatly enriched with our extraction approach.

Plasma membrane-associated proteins that differed between HCC and corresponding normal tissue samples

Coomassie-stained gels containing HCC and control normal tissue lysates had averages of 758 and 713 spots, respectively (Fig. 2). Thirteen plasma membrane-associated proteins were successfully identified; most of them have been described to be involved in cellular functions during carcinogenesis, including adhesion, proliferation, apoptosis, signal transduction, and cytoskeletal remodeling (Table 2). Of these, RhoA was one of the top hits, displaying a more than fivefold increase in HCC compared with normal liver tissue (Fig. 3a, b). Therefore, we hypothesized that RhoA could play important roles in HCC and therefore focused our attention on this protein. Western blotting showed that RhoA was overexpressed in HCC tissues compared to normal tissues (Fig. 3c), consistent with the results from the 2-DE analysis.

Overexpression of RhoA was associated with poor differentiation and metastasis

To further evaluate the potential diagnostic value of RhoA and investigate its oncogenic properties in HCC, immunohistochemistry was carried out to examine the pattern of RhoA expression pattern in clinical tissue samples. In normal liver tissues, weak positive staining of RhoA was detected at the plasma membrane and in the cytoplasm. In 72 HCC specimens, weak (29.2%), moderate (40.3%), and strong (30.5%) positive staining was detected in well, moderately, and poorly differentiated cancer tissues, respectively (Fig. 4, Table 1).



**Table 2** The differential plasma membrane-associated proteins identified by proteomics between HCC and normal liver tissues

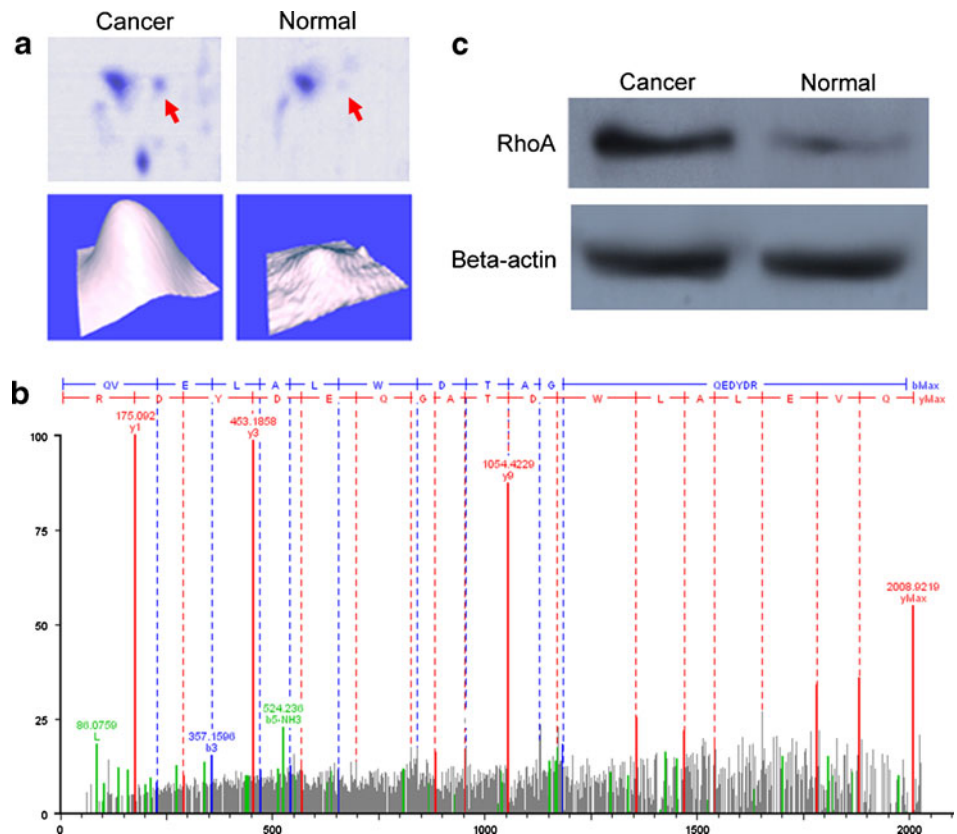
Spot	Protein name	Description	Change
1	Calmodulin	Calcium binding; signal transduction	+7.8
2	CD59 glycoprotein	Inhibition of complement membrane attack	+7.2
3	Transforming protein RhoA	Signal transduction; proliferation	+5.8
4	Annexin A2	Calcium binding; signal transduction	+5.6
5	LIM	Regulation of cytoskeleton and ion channels; migration	+4.5
6	Stomatin-like protein 2	Membrane organization; regulation of ion channels; adhesion	+4.7
7	Protein disulfide-isomerase	Disulfide bond rearrangement; migration	+3.8
8	Moesin	Connection of cytoskeleton to plasma membrane; adhesion	+4.8
9	Ezrin	Connection of cytoskeleton to plasma membrane; adhesion	+8.6
10	Integrin beta-1	VCAM1 and vitronectin receptor; adhesion and migration	+9.1
11	Programmed cell death protein 6	T-cell receptor-, Fas-, and glucocorticoid-induced cell death	-5.3
12	Protein NDRG1	Repression by N-myc; growth inhibition	-5.1
13	Nicastrin	Gamma-secretase cleavage	-5.2

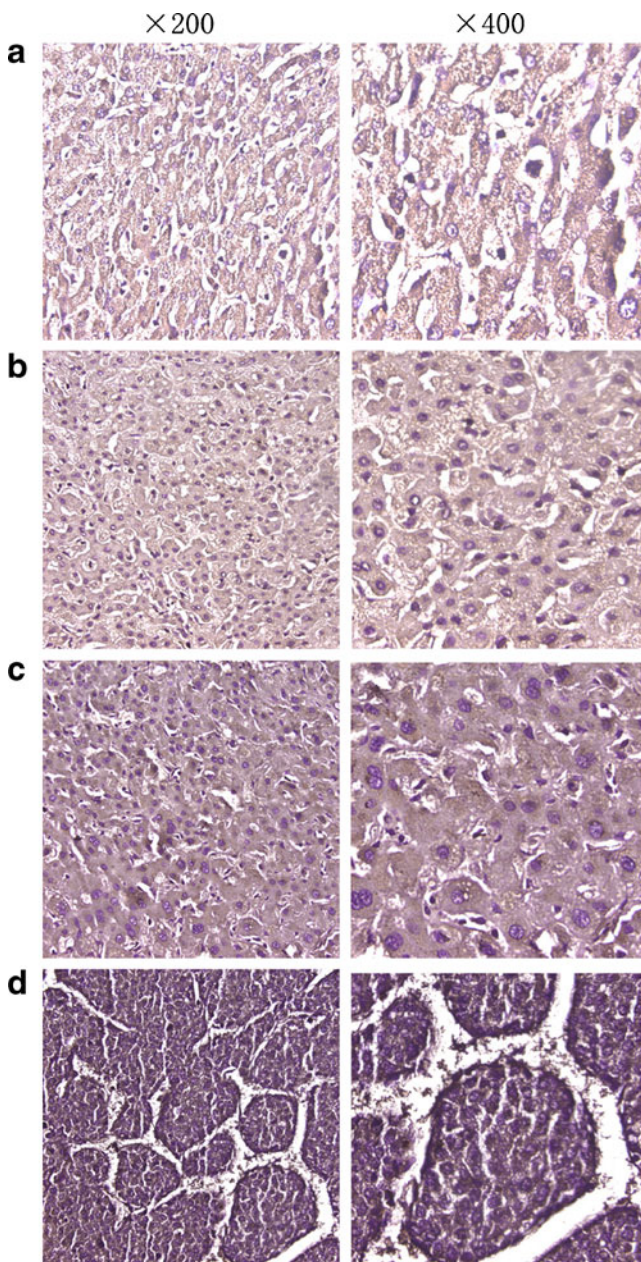
The symbols “+” and “-” denote upregulation and downregulation in HCC, respectively

The clinicopathological correlation of RhoA staining with various HCC parameters was also evaluated, which included gender, age, differentiation, tumor nodules, lymph node involvement, distant metastasis, and clinical stage (Table 1). The quantity and extent of RhoA staining

were significantly increased as tissue differentiation decreased ( $p=0.03$ ). Additionally, RhoA staining was stronger in stage III and IV tissues than in stage I and II tissues ( $p<0.01$ ). It is worth noting that there was no significant difference in RhoA staining among the four

**Fig. 3** Identification of RhoA overexpression in HCC using proteomics. **a** Cropped images from a 2-DE gel (upper panel) and three-dimensional images (lower panel, PDQuest) showing overexpression of RhoA in HCC tissues. **b** Tandem mass spectrometry map for RhoA identification. **c** RhoA overexpression in HCC was validated by Western blotting





**Fig. 4** Immunohistochemical analysis of RhoA expression in clinical HCC tissues. The staining intensities of RhoA increase markedly as tissue differentiation decreases. **a** Normal liver, weak positive staining; **b** well-differentiated HCC, weak positive staining; **c** moderately differentiated HCC, moderate positive staining; **d** poorly differentiated HCC, strong positive staining

T stage groups, though it was significantly different in both the N stage ( $p < 0.01$ ) and M stage ( $p < 0.01$ ) groups. There was no significant difference between the male and female groups. However, RhoA staining had a negative correlation with age. In view of the overexpression of RhoA in poorly differentiated and metastatic HCC tissues, we next investigated its oncogenic properties in vitro using siRNA.

#### Suppression of RhoA in HepG2 cells decreased proliferation and induced apoptosis

SiRhoA-1, which targets RhoA expression, was first applied to HepG2 cells. Western blotting revealed that RhoA expression was reduced by 11%, 62%, and 96% at 72 h post-transfection with 1, 10, and 100 nM siRhoA-1, respectively (Fig. 5a). To observe changes in cellular characteristics, 10 and 100 nM siRhoA-1 were used for further experiments. MTT assay showed that cell proliferation was suppressed by siRhoA-1 (10 nM) in a time-dependent manner and that cell viability was decreased to approximately 50% of the siNC control level at 72 h after transfection (Fig. 5b). Colony formation assay showed that the colony number was reduced by 71.6% in the siRhoA-1 group versus the siNC group (Fig. 5c). These results demonstrate that RhoA suppression leads to a significant decrease in proliferation in HepG2 cells, reflecting the oncogenic capability of RhoA in HCC.

We further investigate changes in cellular characteristics at a higher dose of siRhoA-1 (100 nM). TUNEL assay showed a significant increase in apoptosis in the siRhoA-1 group (Fig. 5d). Hoechst staining clearly showed that the nuclear morphology had apoptotic characteristics such as chromatin condensation and segregation (Fig. 5e). These data demonstrated that apoptosis could be specifically induced by oversuppression of RhoA in HepG2 cells.

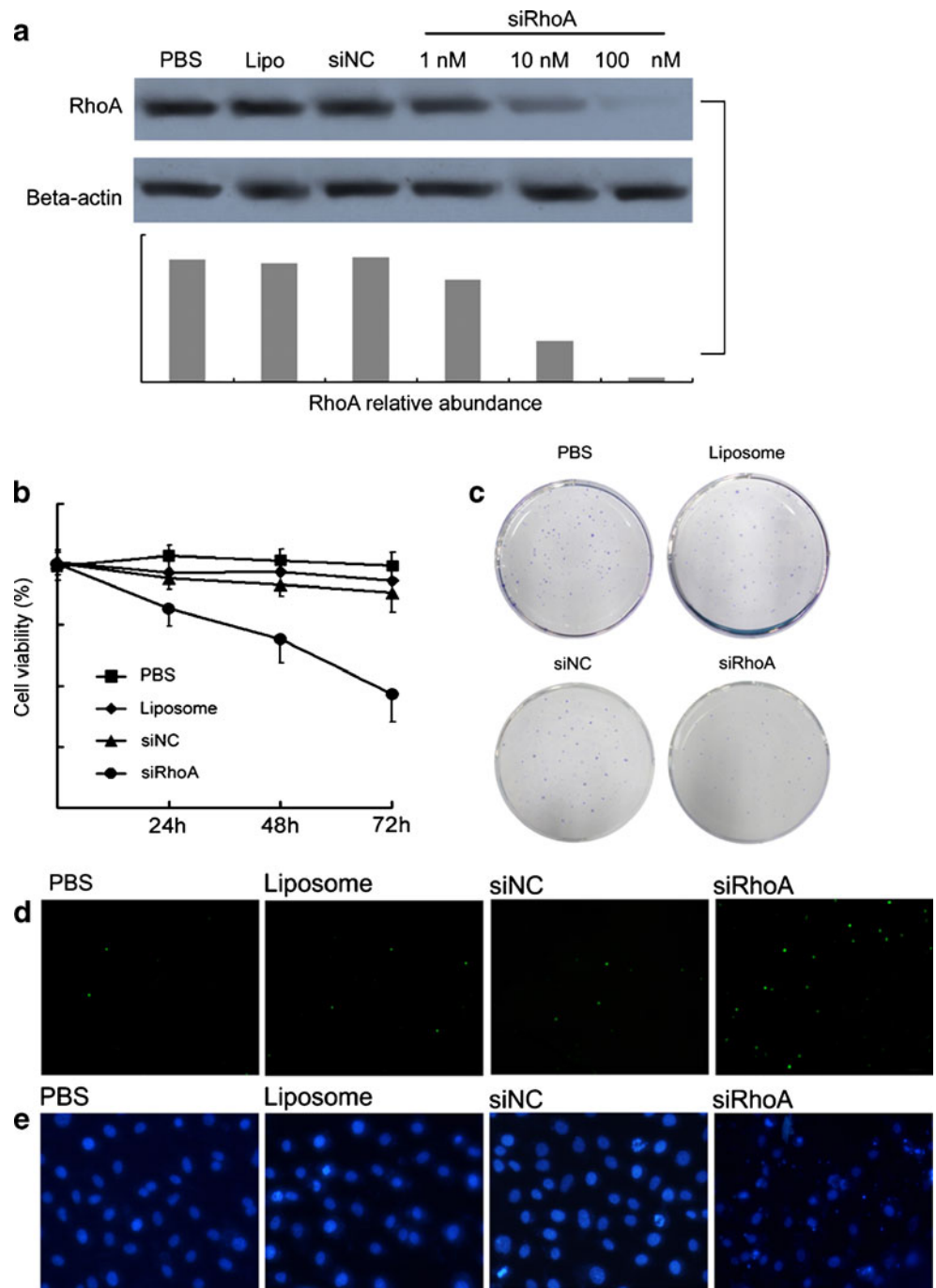
#### Suppression of RhoA in HepG2 decreased cell migration

Since our IHC analysis suggested a close association between RhoA overexpression and HCC metastasis, we investigated cell migration following siRhoA-1 treatment (10 nM). Transwell assay showed that the number of the cells migrating from the upper to the lower well was significantly decreased compared to control (Fig. 6a). Wound healing assay showed that siRhoA-1 group had delayed migration, with cells loosely associating (Fig. 6b). These data demonstrate that suppression of RhoA did indeed decrease cell migration in vitro.

#### Suppression of RhoA in Hep3B cells is associated with growth inhibition and a reduction in cellular migration

To verify the effects caused by RhoA suppression in HepG2, we further performed a set of experiments in Hep3B, a human hepatoma cell line expressing hepatitis B surface antigen. Another siRNA construct against RhoA, termed siRhoA-2, was also used to eliminate the potential off-target effects of siRhoA-1. Western blotting showed that the RhoA expression was reduced by 68% (10 nM siRNA treatment) and 97% (10 nM siRNA treatment) at 72 h post-transfection, respectively (Fig. 7a). MTT assay showed that cell proliferation

**Fig. 5** Suppression of RhoA is correlated with inhibition of proliferation and induction of apoptosis in HepG2 cells. **a** Western blotting confirmed that RhoA expression was inhibited in cells treated with siRhoA-1 (10 and 100 nM). **b** MTT assay showed that siRhoA-1 (10 nM) decreased cell growth in a time-dependent manner. **c** Colony formation assay showed that the number of colonies was decreased with siRhoA-1 (10 nM) after 14 days in culture. **d** TUNEL assay ( $\times 100$  magnification) showed that the number of apoptotic cells in the siRhoA-1 (100 nM) treatment group was significantly increased. **e** Hoechst staining ( $\times 400$ ) revealed evident chromatin condensation and segregation following siRhoA-1 (100 nM) treatment



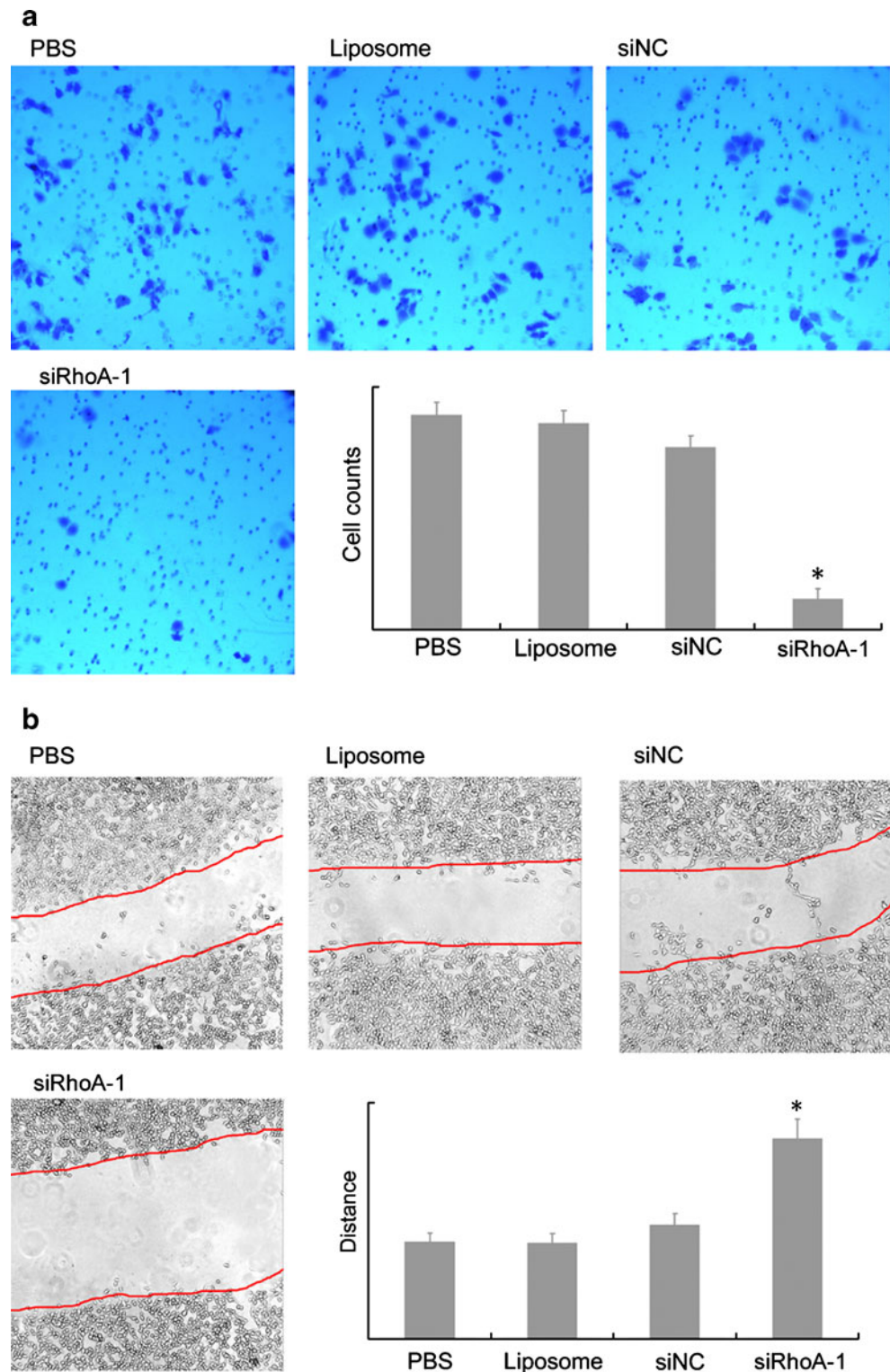
was suppressed by siRhoA-2 (10 nM) in a time-dependent manner and that cell viability was reduced by at least 40% at 72 h after transfection (Fig. 7b). Flow cytometry showed that the number of apoptotic cells in the siRhoA-2 (100 nM) group was significantly higher (24.3%) than that in siNC control group (Fig. 7c). Transwell assay showed that cell migration was reduced by more than 80% after treatment with siRhoA-2 (10 nM). These results in Hep3B cells are similar to those seen in HepG2 cells, indicating that RhoA plays similar roles in both hepatitis B virus (HBV)-negative (HepG2) and HBV-positive (Hep3B) cells.

**Discussion**

Identification of pathology-specific plasma membrane-associated proteins is a key step towards the discovery of potential biomarkers and therapeutic targets for cancer. However, plasma membrane-associated proteins are hydrophobic and expressed less abundantly than cytoplasmic proteins, presenting difficulties for the satisfactory preparation of plasma membrane-associated proteins. In the present study, we prepared plasma membrane-associated proteins using a strategy combining sucrose density gradient centrifuri-



**Fig. 6** Suppression of RhoA is correlated with migration in HepG2 cells. **a** Transwell assay showed that cell migration was decreased upon siRhoA-1 (10 nM) treatment ( $*p < 0.01$ ). **b** Scratch assay showed that cells treated with siRhoA-1 (10 nM) had a delay in migration, with cells loosely associating compared with the three control groups ( $*p < 0.01$ )

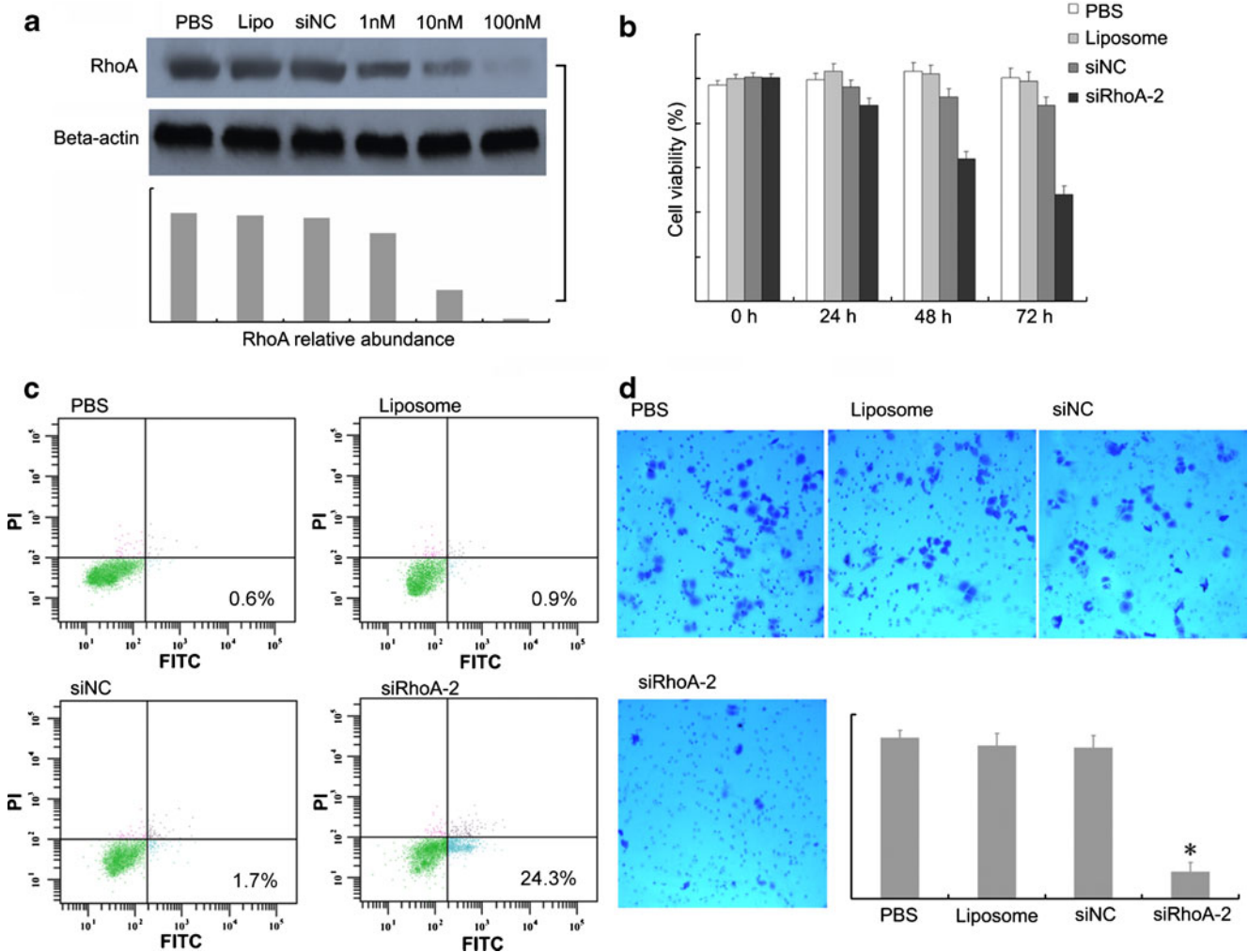


gation and subsequent phase partition and compared the proteomic profiles of HCC and normal liver tissues by means of a 2-DE and mass spectrometry-based proteomics approach.

We focused on the subset of plasma membrane-associated proteins instead of the complete proteome because many plasma membrane-associated proteins play

important roles in tumor development and progression [25, 26]. The enrichment of plasma membrane-associated proteins that is possible with our purification strategy increases the likelihood of finding valuable biomarkers on the cell surface. Although sucrose density gradient centrifugation is a classic method for enriching plasma mem-





**Fig. 7** RhoA suppression in Hep3B cells is associated with inhibition of growth and migration. **a** Western blotting confirmed that RhoA expression was inhibited in cells treated with siRhoA-2 (10 and 100 nM). **b** MTT assay showed inhibition of cell growth in a time-dependent manner after treatment with siRhoA-2 (10 nM). **c** Flow

cytometric analysis showed an obvious increase in apoptosis induced by siRhoA-2 (100 nM). **d** Transwell assay showed the migration ability of cells was decreased following siRhoA-2 (10 nM) treatment (\**p*<0.01)

brane proteins, obtaining reproducible, satisfactory purification using this method is often troublesome, likely because of the similar densities of the plasma membrane and endomembranes such as the mitochondrial membrane and endoplasmic reticulum membrane [27, 28]. Therefore, we further adopted the use of phase partition after gradient centrifugation to enrich plasma membrane-associated proteins. This method of phase partition is based mainly on the principle that plasma membrane and endomembranes possess different charges, which leads to their different distribution coefficients in two-phase solution [20]. In order to assess the quality of the purification, we investigated the levels of marker proteins for the plasma membrane, mitochondrial membrane, and endoplasmic reticulum membrane. Immunoblotting revealed that the plasma membrane-associated proteins were greatly enriched with our com-

bined purification strategy; thus, our high-quality approach provides the best chance to identify plasma membrane-associated proteins of interest.

A total of 13 differentially expressed plasma membrane-associated proteins were identified, all of which were involved in various biological processes related to carcinogenesis such as adhesion, proliferation, signal transduction, and cytoskeletal remodeling. Comprehensive analysis of the differences in the plasma membrane levels of these proteins in the HCC versus normal liver tissues could yield interesting data regarding HCC initiation and development. Of the 13 proteins identified, RhoA aroused our interest because it is highly expressed in HCC and plays interesting roles. RhoA belongs to a small GTPase superfamily that includes the Ras, Rho, Rab, Arf, and Ran subfamilies [29, 30]. As a member of the Rho subfamily, RhoA is mainly

involved in the formation of stress fibers and focal adhesions and is essential to the maintenance of cell polarity as well as to intercellular junctions, cell movement, and migration [31, 32]. Recent studies have shown that RhoA can regulate cell proliferation and apoptosis and may be involved in the carcinogenesis of multiple malignant tumors including lung, breast, colon, ovarian, and prostate cancer [33, 34].

To the best of our knowledge, this study is the first proteomic report showing association of RhoA with HCC. In this work, proteomics was used as a preliminary screening tool for the identification of interesting proteins, and our final objective was to discover potential biomarkers with clinical significance. Consequently, it is necessary to translate these laboratory findings to clinical applications such as diagnosis and treatment. Therefore, a library composed of 72 clinical tissue samples was studied using immunohistochemistry to further assess the clinical significance of RhoA expression. The RhoA staining intensities in normal liver tissues and well, moderately, and poorly differentiated HCC tissues increased in a marked manner that paralleled the loss of differentiation. The close relation between the increased expression of RhoA and poor differentiation and metastasis indicated that RhoA could be used as an independent or supplementary biomarker for predicting HCC prognosis.

The biological functions of RhoA in cell proliferation and migration were investigated in vitro using RNA interference. Taking into account the fact that some cases of HCC are accompanied by infection with HBV, two cell lines, the HBV-negative HepG2 and the HBV-positive Hep3B, were used in this study. The similar results we obtained in the two different cell lines indicate that RhoA play similar roles regardless of HBV infection status. The fact that suppression of RhoA was sufficient to inhibit cell growth reflects the oncogenic properties of RhoA in HCC. In addition, the decrease in migration caused by RhoA suppression suggests that RhoA is involved in HCC migration, which correlates with the observation that higher RhoA expression was found in tissues from patients with lymph node and distant metastasis. These in vitro findings suggest that RhoA could be a potential therapeutic target in HCC and could be inhibited via treatments such as gene, antibody, and chemical therapies.

In summary, our data present a role for RhoA in HCC tumorigenesis through a combination of proteomic analysis, pathological validation, and cell function analysis. We demonstrated that RhoA overexpression was associated with poor differentiation and metastasis in HCC. We also found that RhoA suppression inhibited cell growth and migration in vitro. Our findings strongly suggest that RhoA is a promising biomarker for HCC and possibly a therapeutic target for HCC treatment.

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**Disclosure of potential conflict of interests** The authors declare no conflict of interests related to this study.

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