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An in vitro study to explore the role of prolylcarboxypeptidase in non-small cell lung cancer

Binbin QIAN[1]; Xiaoduo LIU[2]; Xiaolin GU[1]; Lu YANG[3]; Dake CHEN[1,*]

[1] Department of Oncology, Nantong Tongzhou People's Hospital, Nantong, 226300, China
[2] Department of Laboratory Medicine, Nantong Tongzhou People's Hospital, Nantong, 226300, China
[3] Department of Obstetrics and Gynaecology, Nantong Tongzhou People's Hospital, Nantong, 226300, China

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Abstract: Prolylcarboxypeptidase (PRCP) belongs to the S28 family of proteases, which is also a dipeptidyl peptidase. In this study, we demonstrate the expression pattern of PRCP in Non-small cell lung cancer (NSCLC). We found that the repression of PRCP expression by small interfering RNA successfully inhibited cell proliferation, migration, and invasion. Further, we explored the involvement of PRCP in the regulation of epithelial-mesenchymal transition (EMT). The epithelial marker E-cadherin was significantly increased, meanwhile mesenchymal markers MUC1, vimentin, and SNAIL were markedly decreased in PRCP knockdown cells. Moreover, the downregulation of PRCP in the NSCLC cells induced the expression of apoptosis-related proteins in vitro. We performed RT-PCR in 30 pairs of clinical NSCLC tissues and adjacent non-cancerous tissues, which revealed significantly higher PRCP expression levels in cancer tissues than in adjacent non-cancerous tissues. Collectively the results from our study suggest a possible cancer promotion role of PRCP in NSCLC.

Introduction

Lung cancer presents with a very high incidence of morbidity as well as mortality among all cancers, especially metastatic cancers, and this remains the prominent etiology for cancer-related death globally (Siegel *et al*., 2017). The lung cancer is further classified into small-cell lung cancer and non-small-cell lung cancer (NSCLC), where the latter accounts for about 85-90% of the total lung cancer reported (Owonikoko *et al*., 2007; Novello *et al*., 2016). Even with improved diagnostic techniques and advanced treatments, the prognosis remains disappointing. The 5-year survival rate estimation is considered as the survival index following any treatment for cancers, unfortunately, in the case of NSCLC it is just 15%, which even declines to 5% if it happens with metastatic cancer (Rami-Porta *et al*., 2014). The increasing cases of recurrence and vulnerability for metastasis are the underlying cause of failure of treatment in NSCLC patients (Ma *et al*., 2017). Moreover, a significant increase in mortality was observed in patients identified to have a higher N stage. Specifically, in the case of NSCLC patients with distant organ metastasis, lymph node metastasis was associated with a higher chance of experiencing multiorgan metastasis and poor prognosis (Yang *et al*., 2019). The current treatment available for NSCLC includes surgery, chemotherapy, radiation therapy, and target cell therapy. Nevertheless, the high rate of recurrence and the advanced stage while identifying the disease for the first time, it is highly necessary to explore and find out the radical cause for the development and progression of NSCLC at the earliest. Smoking is perceived as one of the most important risk factor leading to various types of cancers. Many clinical studies have proved that the clinical manifestation, as well as prognosis among smokers and non-smokers, are different (Lee *et al*., 2014; Muallaoglu *et al*., 2014).

Prolyl carboxypeptidase (PRCP), also known as angiotensinase C, is an enzyme with many significant functions. This particular enzyme has been recognized to sustain an internal environment to maintaining hemodynamic stability at the tissue level, and it is associated with preeclampsia, rheumatoid arthritis, and tonsillitis (Mallela *et al*., 2009). PRCP belongs to the S28 family of proteases and is a dipeptidyl peptidase. Nonetheless, it contributes very limited sequence integrity with other proteases, it is capable to generate mature lysosomal prolyl carboxypeptidase (Abeywickrema *et al*., 2010). The location of the PRCP gene is in chromosome 11. At first, it was identified in the kidney of swine (Yang *et al*., 1968), and later cloned from a human kidney library. PRCP expression has been recognized in the brain, lung, heart, liver, and adipose tissue, which suggests multiple organ-specific roles. Moreover, it has been detected in lysosomes and extracellular matrix either as in the form of membrane-bound or as a soluble form (Miller *et al*., 1991). It was identified to inactivate angiotensin II as well as angiotensin III, and also to generate plasma kallikrein from prekallikrein with the subsequent production of bradykinin, and abridge the anorexigenic

*Address correspondence to: Dake Chen, 15962852220@sohu.com

neuromodulator melanocyte-stimulating hormone (MSH) (Moreira *et al*., 2002; Shariat-Madar *et al*., 2002; Wallingford *et al*., 2009). Some studies have shown that PRCP has an affinity towards the amino acid sequences (Kumamoto *et al*., 1981). These biological properties have been recognized to affect the blood flow as well as variations in the blood pressure, inflammatory responses, and vary the regulation of appetite. Henceforth PRCP has been implied for the treatment of obesity, and clinical studies identified that the downregulation of MSH by PRCP leads to increased food intake, which in turn leads to obesity (Shariat-Madar *et al*., 2010). It is believed that the site of this effect possibly is in the neurons of the hypothalamus (Zhou *et al*., 2010). Recently many studies revealed that the dysfunction of PRCP can lead to adverse cardiovascular disorders such as inflammation and hypertension (Tamaoki *et al*., 1994). Genetic studies have found that PRCP is recognized for the progression of metabolic syndromes (Watson *et al*., 1997; McCarthy *et al*., 2003). A study conducted among the African-American patient population revealed that a particular exon SNP identified as rs2298668, E112D of PRCP is associated with the possibility of chronic hypertension and preeclampsia (Wang *et al*., 2006). Likewise in a Chinese patient population, PRCP was identified to outturn treatment resistance to an anti-hypertensive agent, Benazepril (Zhang *et al*., 2009). Despite the ambiguous physiological perspective of PRCP, it has been observed to have an active role in cell permeability through the activation of prekallikrein and the melanogenic signaling pathway (Mallela *et al*., 2009). However, the biological distinctiveness of PRCP in NSCLC remains to be explicated. This study aimed to illustrate the expression pattern of repressed PRCP in NSCLC cell lines.

Materials and Methods

Collection and handling of tissue samples

The samples were collected from 30 randomly selected individuals with confirmed NSCLC. All NSCLC tissues and adjacent normal tissues were carefully handled for formalin-fixation and further paraffin embedding. The patients included in this study were prior to surgery, and proper consents were obtained from all patients. We collected and frozen all samples. The lower age limit of these patients was 41.2 years, and the higher limit was 60.3 years, with the median age of 51 years, which included 12 women and 18 men. The clinical characteristics of each patient were clarified with their case records and confirmed that none had received either radiotherapy or chemotherapy prior to the surgery. The clinical biobank of the Affiliated Hospital of Nantong University, Jiangsu Province, China, provided the samples for this study. This study was recognized for it strictly carried out the procedures for care and use admitted by the Ethics Committee of the Affiliated Hospital of Nantong University (Ethic Number: 2018-K020).

Treatment of cells

NSCLC cell lines namely SPC-A-1, NCI-H1975, A549, and NCI-H1650 were collected from the Cell Bank, Type Culture Collection, Chinese Academy of Science (CBTCCCAS, Shanghai, China). Immediately upon arrival of the cells, we transferred them to culture bottles along with RPMI 1640 and 10% fetal bovine serum (FBS) as the nutrition medium, 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin, and placed it in a chamber with 5% CO_2 humidified atmosphere at a temperature of 37.8°C.

Detection of RNA expression by RT-PCR

The entire RNA was extracted using RNAiso Plus (TaKaRa, Japan) and reverse transcribed into cDNA with the Prime Script RT Reagent Kit (TaKaRa, Japan). Then we proceeded RT-PCR with ABI 7500 FAST Real-Time PCR System (Applied Biosystems, Carlsbad, USA) and SYBR Green Master Mix (Takara, Dalian, China). The quantification of gene expression levels was obtained using 2^{2DDCt} method standardization to the endogenous reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH). To avert inaccuracy, RT-PCR was performed thrice. The primers used for PCR amplification were as follows: PRCP forward: 5'-TCTACACTGGTAATGAAGGGGAC-3', PRCP reverse: 5'-CCCACATGAACCCCGTGTTA-3'; GAPDH forward: 5'-GGTAGACAAGTTTCCCTT-3', GAPDH reverse: 5'-ATATGTTCTGGATGATTCT-3'.

Western blot analysis

Cell lysis was performed using radioimmunoprecipitation assay (RIPA) lysis buffer (P0013B, Beyo-time Institute of Biotechnology, Nantong, China). After a period of 48 h following the process of silencing with shRNA, the estimation for the concentration of protein was performed by the BSA method (Beyotime Institute of Biotechnology). The proportionate volume of protein was detached to 10% sodium dodecyl sulphate (SDS)-polyacrylamide gels, further we performed electrophoresis and obtained the gels containing the markers. Gels were then transplanted to the Polyvinylidene Fluoride Membrane. 5% of non-fat milk was used for membrane blocking and incubated with the specific primary antibody overnight. In the early hours of the following day, membranes were washed thrice with TBST and again incubated with appropriate secondary antibody (Beyotime Institute of Biotechnology) at a dilution of 1:5000 for 1 h at room temperature. The enhanced chemiluminescence (ECL) system (Millipore, Bedford, MA) was used to quantify the protein expression. The primary immunoblotting antibodies were: anti-b-Actin (dilution 1:1000, 4970, Cell Signaling Technology, Danvers, MA), anti-PRCP (dilution 1:1,000, ab42080, Abcam, Cambridge, MA), anti-p53 (Epitomics, Burlingame, CA), anti-BAX (dilution 1:1000, ab32503, Abcam), anti-APAF1 (dilution 1:1000, ab32372, Abcam), anti-MUC1 (dilution 1:1000, ab45167, Abcam), anti-E-cadherin (1:50, ab1416), anti-vimentin (1:1000, ab92547, Abcam).

Transfection and cell lines generation

The silencing of PRCP gene expression was performed using three small interfering RNA (named shPRCP, shRNA-1 sense: shRNA-1:5'-CACCCTCTATTCTATCTCA-3'; shRNA-2 sense: 5'-GAAAATCAAAGTGAATGGG-3'; shRNA-3 sense: 5'-AAGAAAGTAACTCTCCCTC-3') and the negative control duplex (named shControl, sense: 5'-TTCTCCGAACGTGTCACGT-3') with no potential similarity with any known gene were used. Biomics Biotech, Nantong, China, provided RNA duplexes Lipofectamine 2000 reagent (Invitrogen) was used to perform oligonucleotide transfection.

Among the three shRNAs, we have chosen shRNA-1 as the most efficient sequence through RT-PCR as well as western blot. Since shRNA-1 has been observed to have the highest silencing efficiency, we continued the experiment exclusively using shRNA-1. Full-length PRCP cDNA was cloned to generate a pCMV6/AC/GFP vector. Plasmids screening was conducted with Green Fluorescent Protein (GFP) analysis, cell culturing performed with 200 μg/mL G418 (Invitrogen). The expression levels of these plasmids were

observed by RT-PCR. Cell clones were replicated after 30 days. The PRCP expression of cell lines was silenced using shRNA-1 and named SPC-A-1 shPRCP and H1975 shPRCP, and their corresponding controls were named SPC-A-1 shControl and H1975 shControl.

Cell proliferation assay

The cell lines; SPC-A-1, NCI-H1975, A549 and NCI-H1650 were cultured and cells were counted, roughly 5.3×10^3 cells were placed into each well of the 96-well plates for 24 h, the cell transfection was performed using RNA duplex, shPRCP or shControl for 4 days to obtain an ultimate concentration of 50 nM. For every 24 h, the medium was restored with WST-8/CCK-8 (Dojindo Laboratories, Japan), following incubation for an hour at 37.8°C, the rate of absorbance was detected using the spectrophotometer at 450 nm with an MRX II absorbance reader (Dynex Technologies, USA).

Cell migration and invasion assay

Migration and invasion assay was performed using Transwell chambers (Millipore). The inserts were pre-coated with Matrigel (BD Biosciences, USA) on the upper surface and placed into the culture plate. Following successful transfection, approximately 8.3×10^4 cells from each group were transferred into the medium (0.2 mL), which did not contain any nutrition agent added to the upper surface. Followed by adding 0.6 mL RPMI-1640 containing 10% FBS to the lower compartment. After incubating for 24 h at 37.8°C, cells resting on the upper surface were removed and immersed the lower compartment into 100% methanol and stained using a 0.3% crystal violet solution. For determining the number of cells invading and migrating, we captured five random visual fields (maximum magnification, 3200) of each insert, and cells were counted under the light microscope (Olympus, Japan).

Statistical analysis

All data were expressed as the mean ± standard deviation (SD). $\chi 2$ test or two-tails test was implemented to calculate the significance between the groups. SPSS16.0 software (IBM, USA) was used for data analysis. Data were considered significant with $p < 0.05$.

Results

PRCP expression levels

RT-PCR was performed with 30 random pairs of NSCLC tissues and surrounding normal tissues. We have noticed remarkably higher PRCP expression levels in NSCLC tissues than in surrounding normal tissues (N = 30; Fig. 1(A)). Furthermore, we investigated the expression level of PRCP mRNA as well as PRCP protein in the following NSCLC cell lines (SPC-A-1, A549, H1975, and H1650). Each cell line demonstrated a distinct level of expression (Fig. 1(B–D)), the highest expression of PRCP was displayed in SPC-A-1 and H1975 cells, followed by A549 and H1650, both at mRNA as well as protein levels.

Cell transfection

Since SPC-A-1 and H1975 were detected to have high PRCP expression levels, these two cell lines have been transfected with shPRCPs (shRNA-1, shRNA-2, and shRNA-3), along with negative control shRNA and normal cells (Fig. 2(A)). The western blot analysis proved lower PRCP protein expression, and RT-PCR proved lower mRNA expression in shPRCP-transfected cell lines (Fig. 2(B)). The results from the western blot and RT-PCR revealed that shRNA-1 had superior silencing efficiency in both protein and mRNA levels. Therefore shRNA-1 alone was utilized in the loss-of-function studies.

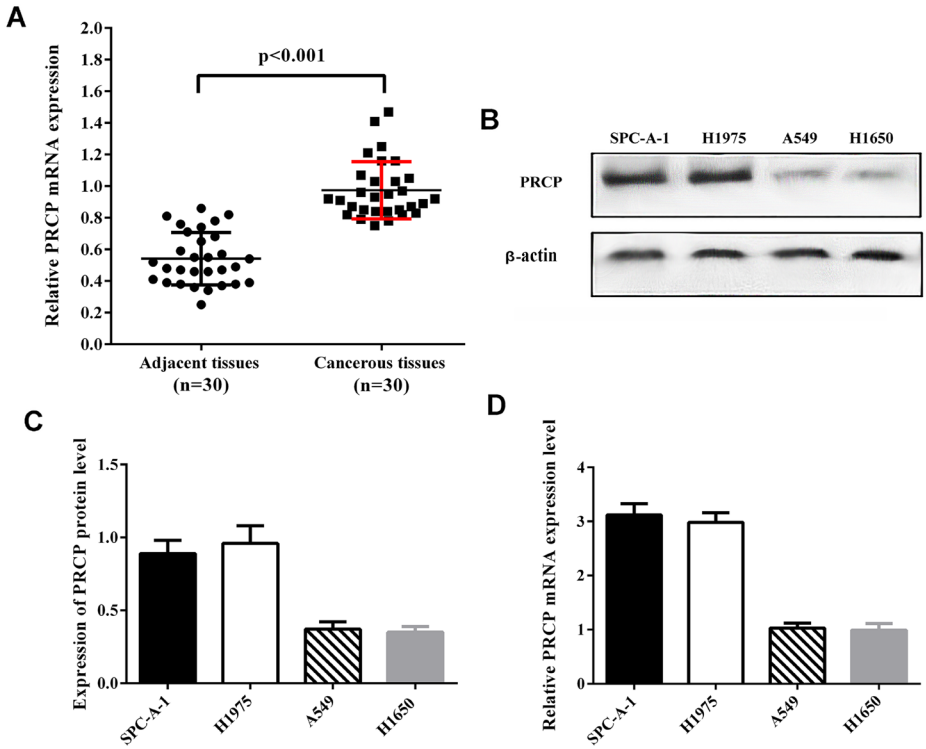

FIGURE 1. The relative expression of PRCP in NSCLC tissues and cell lines. (A) The relative PRCP expression levels in 30 random pairs of NSCLC tissues. (B) Results of western blot analysis to identify the cell line exhibiting highest PRCP expression level among the four NSCLC cell lines. (C) Demonstration of the protein expression level of PRCP in four NSCLC cell lines SPC-A-1, H1975, A549, and H1650. (D) Demonstration of the mRNA expression level of PRCP in four NSCLC cell lines SPC-A-1, H1975, A549, and H1650.

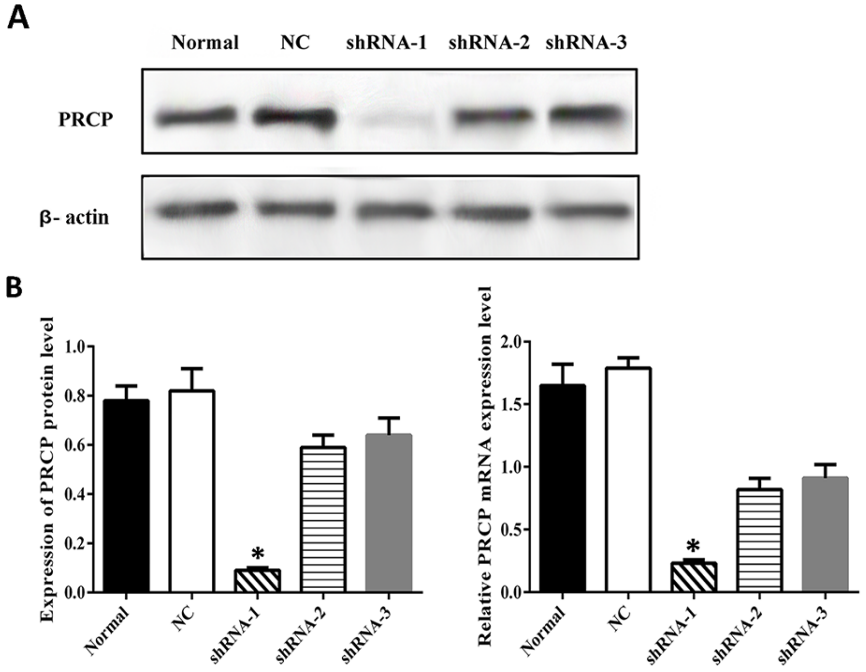

FIGURE 2. Detection of the most efficient shRNA for silencing the expression of PRCP. (A) Results of western blot analysis to identify the PRCP expression in three different sh-transfected groups shRNA-1, shRNA-2, shRNA-3, normal and negative control. (B) Relative protein expression and relative mRNA expression in shRNA-1, shRNA-2, shRNA-3, normal and negative control, respectively.

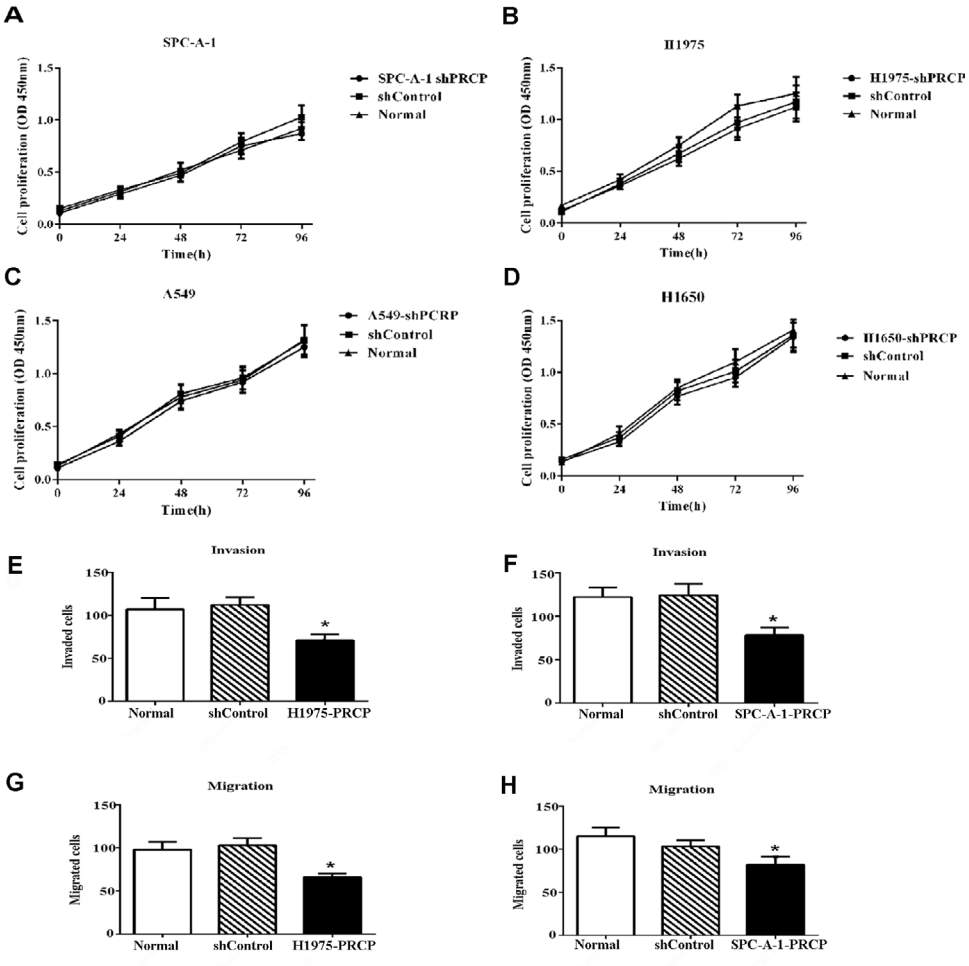

FIGURE 3. Effect of PRCP in regulating NSCLC cells proliferation and motility in vitro. Graphical representation of CCK-8 assay to demonstrate the proliferation ability of NSCLC cells by the repression of PRCP expression using shPRCP in (A) SPC-A-1, (B) H1975, (C) A549, and (D) H1650 cells respectively. Graphical representation of Transwell assay to demonstrate the cell invasion and migration in shPRCP-transfected cells compared with control cells (E) and (G) H1975-PRCP, (F) and (H) SPC-A-1-PRCP, respectively.

Inhibition of cell proliferation, migration and invasion of NSCLC cells in vitro

SPC-A-1 and H1975 cell lines were identified to have high PRCP expression levels among all the four cell lines. Therefore PGPH1/GFP/Neo vectors consisting of PRCP shRNA-1 were used to silence the PRCP gene expression of SPC-A-1 as well as H1975. Two potent PRCP transfected cell lines were developed using G418. Further, we investigated the effect enhanced by PRCP on cell proliferation using Cell Counting Kit-8 (CCK8) assay, knockdown of PRCP expression by shPRCP inhibited the proliferation capacity of transfected cells when compared with the control cells as time advanced (Figs. 3(A)-3(D)). Then we explored the impact of PRCP in the invasion and migration of SPC-A-1 and H1975 cells. Results of the matrigel invasion assay revealed, significant reduction of the invasiveness in shPRCP-transfected cells H1975 and SPC-A-1 respectively (Figs. 3(E)–3(F)), At the same time the Migration assays also proved that the cell motility was significantly reduced in shPRCP-transfected cells, compared with control cells of H1975 and SPC-A-1 respectively (Figs. 3(G)-3(H)).

Regulation of EMT markers and apoptosis-related proteins by repressed PRCP gene in vitro

We further explored the effects on Epithelial-Mesenchymal Transition (EMT) markers (MUC1, E-cadherin, vimentin, and SNAIL) generated by the cells with repressed PRCP expresssion. Significantly higher protein levels of MUC1, E-cadherin and SNAIL were noticed in shPRCP transfected cells , at the same time the protein level of vimentin was significantly lower compared to their corresponding control cells and normal cells in vitro (Figs. 4(A)-4(C)), except for the low expression of MUC1 in SPC-A-1 as well as for the low expression of MUC1 and SNAIL in H1975 shPRCP cells. Further we examined the impact of repressed PRCP on apoptosis-related proteins (p53, BAX, and APAF1), we were able to detect significant increase in the expression of p53, BAX, and APAF1 in shPRCP-transfected cells compared with their corresponding control cells and normal cells in vitro (Figs. 5(A)-5(C)). Expression levels of proteins were examined to declare the outcomes.

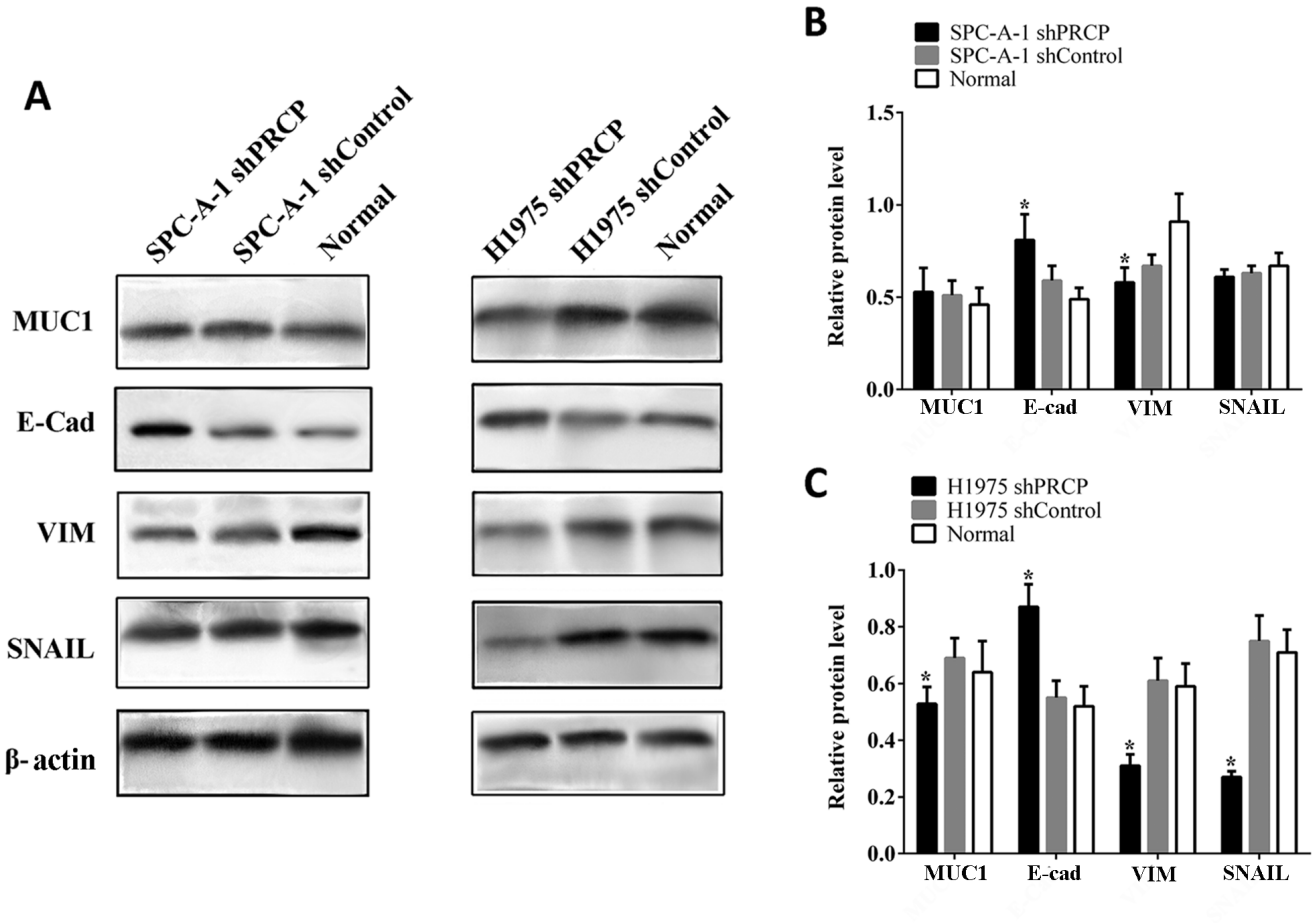

FIGURE 4. Repression of PRCP in regulating EMT markers. (A) Results of western blot analysis to demonstrate the expression levels of EMT markers (E-Cad, MUC1, VIM and SNAIL) in PRCP silenced SPC-A-1 and H1975 cells. (B) Graphical representation of relative protein expression in SPC-A-1 shPRCP, SPC-A-1 shControl and normal cells respectively. (C) Graphical representation of relative protein expression in H1975 shPRCP, H1975 shControl and normal, respectively.

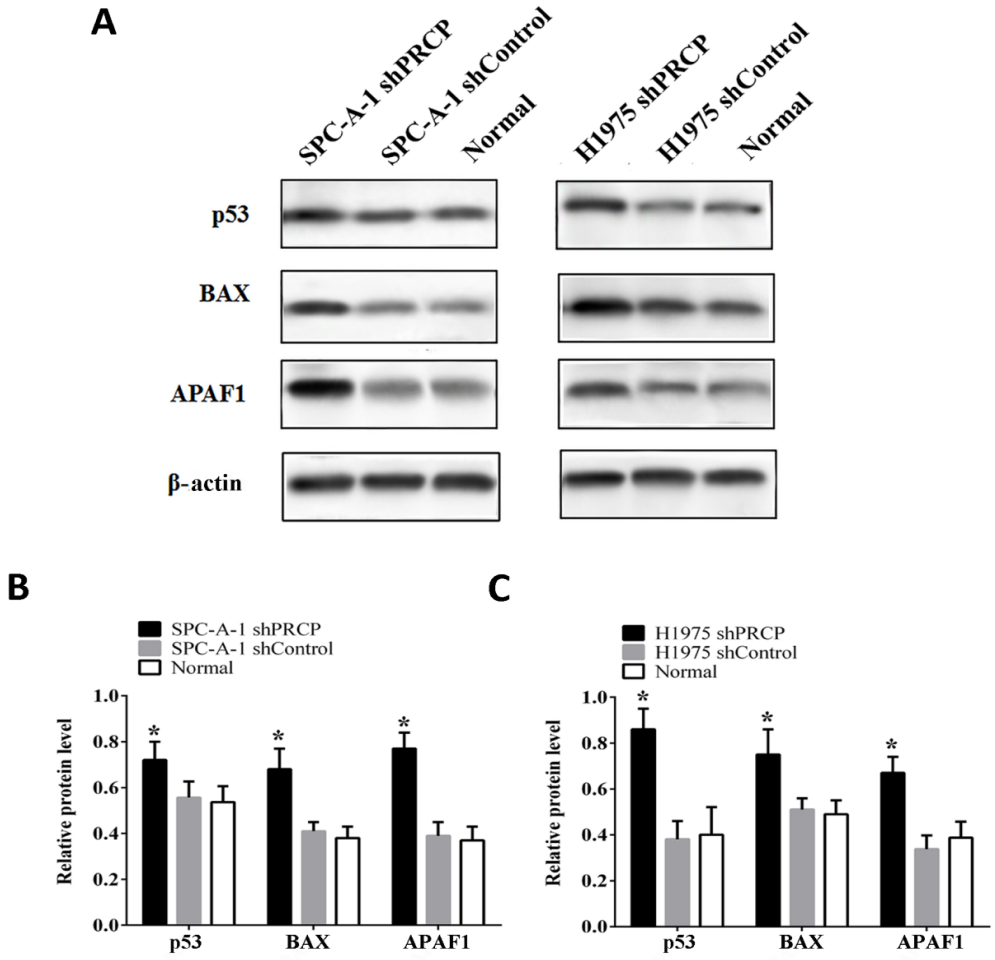

FIGURE 5. Repression of PRCP in regulating apoptosis-related proteins. (A) Results of western blot analysis to demonstrate the expression levels of apoptosis-related proteins (p53, BAX and APAF1) in PRCP silenced cells. (B) Graphical representation of relative protein expression in SPC-A-1 shPRCP, SPC-A-1 shControl and normal cells, respectively. (C) Graphical representation of relative protein expression in H1975 shPRCP, H1975 shControl and normal, respectively.

Discussion

PRCP belongs to the S28 family of proteases, which is also a lysosomal serine carboxypeptidase capable to cleave various C-terminal amino acids adjacent to proline. However, the genetic involvement of PRCP associated with cancer in humans is widely unexplained. Our efforts for this study explain that PRCP expression was significantly higher in NSCLC tissues compared with the adjacent normal tissues, furthermore, we found that the PRCP gene has the potentiality to promote the proliferation, migration, and invasion of NSCLC cell lines in vitro. These results strongly suggest that the PRCP gene is having a cancer promotion role in NSCLC.

At present, various treatment options are available for NSCLC, nevertheless, most of the lung cancers by virtue of high chances of relapse were noticed to evolve resistance to the therapy (Chang, 2011). Radiotherapy is a treatment that involves the transmission of highenergy rays to impair cancerous cells; these rays have a higher magnitude than the rays in conventional x-rays used for imaging purposes. Unfortunately, the successful outcome of radiotherapy is confined as a result of the survival of some cancer cells following the treatment, which results in the recurrence of the disease followed by poor survival of the patient (Willers *et al.*, 2013). Hence, the study and invention of novel therapeutic agents to overthrow such limitations are indispensable for recovery from cancer. The transformation of the normal cells into cancerous cells in NSCLC comprises various factors and involves multiple genes (Xie *et al.*, 2014). The principle mechanism for carcinogenesis is so far considered either as the down-regulation of the so-called tumor-suppressor genes or the up-regulation of certain oncogenes, which is known to involve numerous changes at the cellular, genetic, and epigenetic level. Eventually, it alters the cell division mechanism and leads to metastatic diseases (Lee *et al.*, 2010). PRCP has been observed to downgrade desArg9-bradykinin (B1-receptor agonist), angiotensin II and III, and α-MSH (1–13) (Odya *et al.*, 1978). A recent study by Haley R. Gittleman *et al.* (2016) have demonstrated that PRCP is a plasma prekallikrein activator when prekallikrein accumulates on high molecular weight kininogen on endothelial and other cell membranes; this suggests that PRCP is also an endopeptidase that is capable to break peptide bonds of nonterminal amino acids (Shariat-Madar *et al.*, 2003). Likewise in another Han Chinese patient population, the G allele of intron SNP rs7104980 was found to have an effect on essential hypertension (Wu *et al.*, 2013). Findings from a study conducted by Shengyuan Xu *et al.* among patients diagnosed with myocardial infarction or unstable angina were observed to have increased concentration of PRCP, which suggested an association between cardiovascular

disorder and PRCP. Nevertheless, the underlining principle behind the association is largely unknown; however, increased expression of angiotensin II type 2 receptor in the coronary artery endothelial cells of mice has been identified as the reason for increased expression of PRCP and an increased release of bradykinin (Zhu *et al*., 2010). The serine aminopeptidase DPPII/DPPVII is recognized to share the nearest homologs with PRCP. Therefore, PRCP and DPPII/DPPVII are a pair of proteases showing dissimilarity with other members of the protease family (Mallela *et al*., 2009).

Our results strongly suggest that the knockdown of PRCP expression could inhibit the cell proliferation, migration, and invasion of NSCLC cell lines. Moreover, by analyzing the regulation of proliferation, migration, and invasion, it is evident that the expression of E-cadherin (epithelial marker) was markedly higher with shPRCP-transfected cells. We have observed the loss of E-cadherin during EMT, for this step reflects the capability to regulate cellular dimension as well as shape (Kalluri and Weinberg, 2009; Qiao *et al*., 2011; van Roy and Berx, 2008). Reduced expressions of MUC1, vimentin, and SNAIL (mesenchymal markers) were noticed with repressed PRCP expression. Moreover, our study with apoptosis-related protein detected a significant increase in the expression of p53, BAX, and APAF1, suggesting the knockdown of PRCP could regulate cell apoptosis (Stewart *et al*., 2016; Gao *et al*., 2009). There are various efficient disease markers discovered in recent years and successfully proved favorable for NSCLC treatment (Kannan *et al*., 2001; Wouters *et al*., 2010; Tang *et al*., 2017; Anaz *et al*., 2019). We proudly present this with the hope of a more detailed exploration of the role of the PRCP gene in NSCLC.

Since the repression of PRCP expression successfully inhibited the cell proliferation, migration, and invasion of NSCLC cell lines, it proves that PRCP has some role in regulating the proliferation and motility of NSCLC. We could find a more possible role for PRCP, its involvement in vivo, and whether it could be used as a prognostic marker and much more in the near future. Collectively, this study concludes that PRCP plays an important role in the development of NSCLC. Results of CCK-8 assay proved that the knockdown of PRCP by shPRCP successfully inhibited the proliferation capacity of transfected cells; these results are favorable to confirm that PRCP could regulate cell proliferation in NSCLC. Since knockdown of PRCP successfully suppressed the proliferation, migration, and invasion in NSCLC cells in vitro, it is worthy enough to proceed with a proper and deeper investigation, which could be a beneficial treatment strategy either for limiting the disease progression or treatment.

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Conflicts of Interest

The authors declare that they have no conflicts of interest to report regarding the present study.

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