

Original Article

RNAi-mediated silencing of praline-rich gene causes growth reduction in human lung cancer cells

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Abstract: Lung cancer ranks among one of the most frequent causes of cancer death in the world. Here, we investigated PRR11, one novel gene, with no functional annotation, was found to be over-expressed in lung cancer patients suggesting its potential implication in tumorigenesis. Furthermore, high PRR11 levels predict shorter survival of lung cancer patients. In this study, we investigated the therapeutic potential of PRR11 in lung cancer using the technique of RNA silencing via small interfering RNA (siRNA). Synthetic siRNA duplexes against PRR11 were introduced into 3 lung cancer cell lines, which subsequently resulted in a significant depletion in PRR11 expression in the cells. We found that the targeted depletion of PRR11 caused a dramatic cell cycle arrest followed by massive apoptotic cell death, and eventually resulted in a significant decrease in growth and viability of all 2 lung cancer cell lines. In summary, our study strongly suggests that PRR11 may serve as a potential therapeutic target in human lung cancer.

Keywords: Lung cancer, RNAi, PRR11 depletion, cell growth

Introduction

Lung cancer is the leading site in all malignant tumors, comprising 17% of the total new cancer cases and 23% of the total cancer deaths. It is also the leading cause of cancer death; most patients are diagnosed with advanced disease, resulting in a very low 5-year survival [1-4]. According to recent reports, lung cancer patients in China have exceeded 500000, more than 400000 people died of lung cancer each year, the total number of patients with lung cancer was the leading site in the world and the incidence of lung cancer was increasingly lifted, which caused serious health hazard, but its etiology and pathogenesis have not fully cleared. Therefore improving the early diagnosis and developing effective targeted therapy might have important significance for improving the prognosis of lung cancer [5-9].

Although a sequential series of human cancer associated genes have been discovered in the past several decades, many more unknown oncogenes remain to be identified, especially for lung cancer, the leading cause of cancer deaths in both women and men throughout the

world. Identification and functional analysis of novel potential cancer-associated genes will undoubtedly increase our understanding of carcinogenesis and is of great importance for developing diagnostic, preventive and therapeutic strategies for the treatment and management of cancer [10-14].

We have recently conducted a meta-analysis of several cancer microarray datasets to search for novel deregulated cancer-associated genes. One novel gene, PRR11, was found to be over-expressed in several lung cancer patients. In the present study, we have isolated and identified this novel gene, and demonstrated that this gene played critical roles in both cell cycle progression and lung cancer development.

In the present study, to investigate the therapeutic potential of PRR11 in lung cancer, we used small interfering RNA (siRNA) to specifically knockdown PRR11 in lung cancer cells. Our results have shown that a targeted siRNA depletion of PRR11 resulted in a significant decrease in cell viability, defects in important mitosis processes (failure of cytokinesis) and cell cycle arrest in several human lung cancer cell lines.

Depletion of PRR11 inhibits lung cancer growth

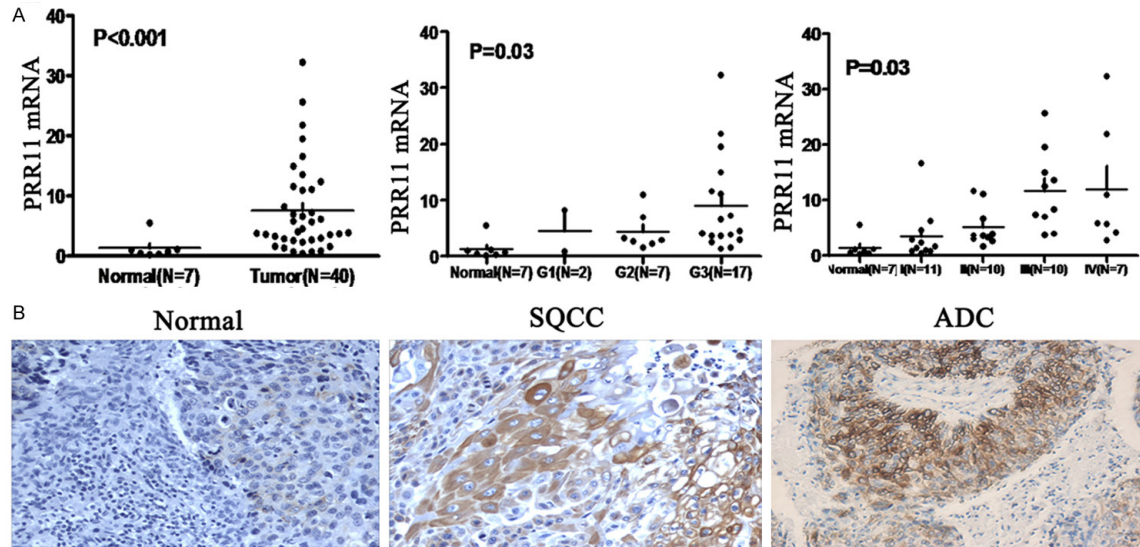


Figure 1. PRR11 is over-expressed in lung cancer. A. Over-expression of PRR11 at mRNA level in lung cancers. Expression of PRR11 in normal and lung cancer tissues was analyzed by qRT-PCR. The grades were classified as G1-G3. The stages were divided into I-IV. N means normal. B. Over-expression of PRR11 at protein level in lung cancers. Expression of PRR11 in normal and cancerous lung tissues was analyzed by immunohistochemical analysis. Representative immunohistochemical results of PRR11 expression in normal and cancerous lung tissues was shown. ADC, adenocarcinoma; SQCC, squamous cell carcinoma.

Materials and methods

Cell culture

Human lung cancer cell lines H1299 and A549 were cultured in DMEM supplemented with 10% FBS and antibiotics. Cells were maintained at 37°C in a water-saturated atmosphere of 5% CO₂ in air. All cells obtained from the cell bank of the Committee on Type Culture Collection of the Chinese Academic of Science (CCTCC, Shanghai, China).

Tissue microarray and immunohistochemistry

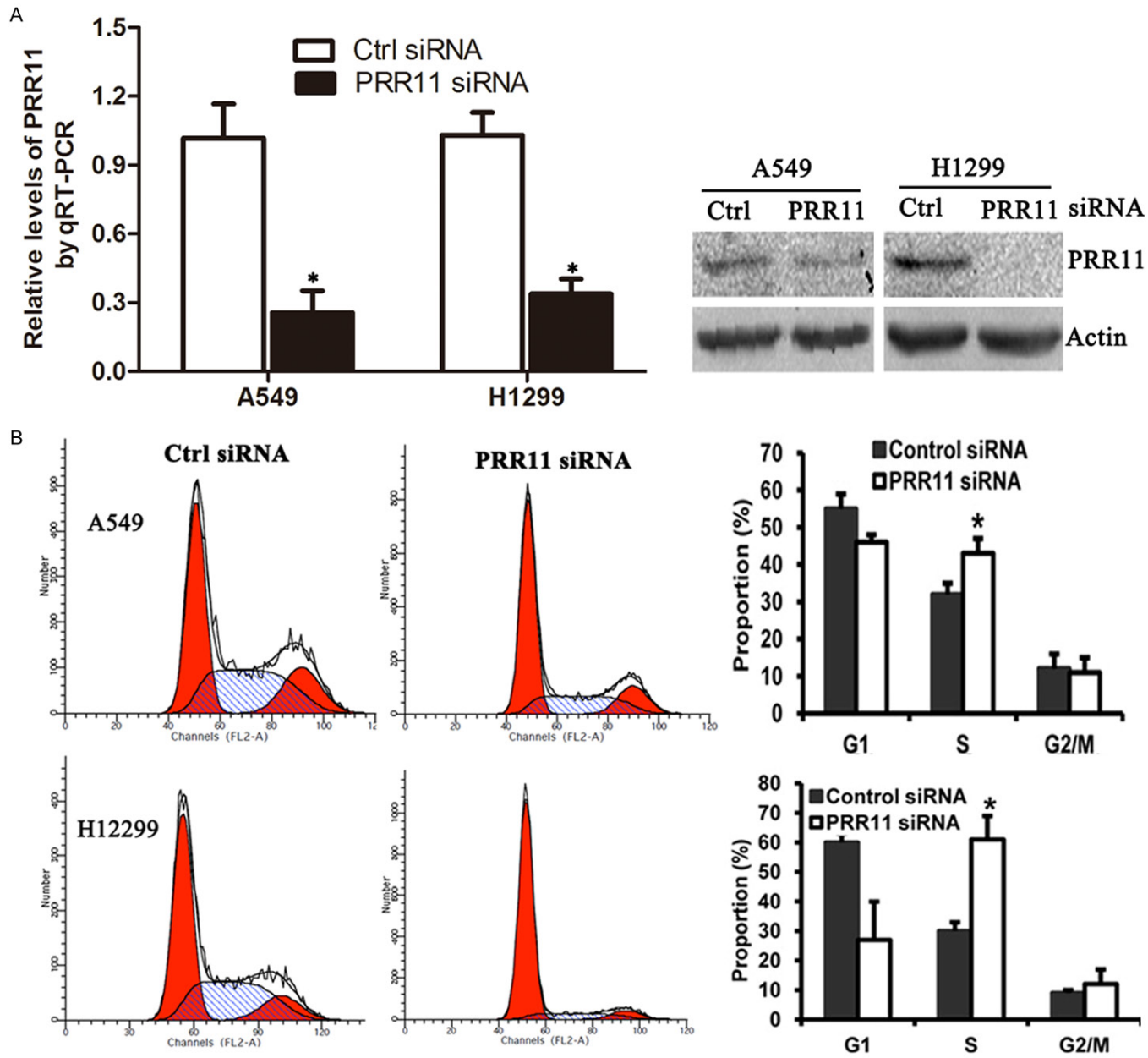
PRR11 protein expression was determined on a lung cancer tissue microarray were purchased from Shanghai Outdo Biotech Company (Shanghai, China). HLug-Squ030PG-01 contains 15 normal lung tissue and 15 lung cancer tissues with duplicated cores. OD-CT-RsLug-01-007 contains 120 lung cancer tissues each with a single core. For immunohistochemistry, slides were routinely deparaffinized and rehydrated, and then were subjected to heat-induced epitope retrieval in 0.01 mM citrate buffer (pH6.0). Endogenous peroxidase activity was blocked for 10 min in 3% hydrogen peroxide and methanol. The slides were then incubated with PRR11 rabbit polyclonal antibody

(1:200, Abcam) at 4°C overnight. Immunodetection was performed using Elivision™ plus Polymer HRP (Mouse/Rabbit) IHC Kit (Maixin. Bio, Fuzhou, China). Negative control was performed using normal rabbit IgG. Based on previous studies, the intensity and extent of the staining were used to assess PRR11 expression. Briefly, the staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong), and the staining extent was scored as 0 (0%), 1 (1 to 25%), 2 (26 to 50%), 3 (51 to 75%), or 4 (76 to 100%), according to the percentages of the positive stained cells in relation to the whole carcinoma area or entire section for the normal samples. The sum of the intensity and extent scores was used as the final staining scores (0 to 7). Tumors having a final staining score of < 3 were considered as low expression and those with scores ≥ 3 as high expression.

SiRNA transfection

The PRR11 targeting siRNA or the control siRNA were purchased from GenePharma (Shanghai, China). The PRR11 siRNA is a mixture of 4 individual siRNAs that target different regions of the PRR11 mRNA, thus ensuring an effective depletion of the PRR11 gene expression in the cells. PRR11 or control siRNA were transfected

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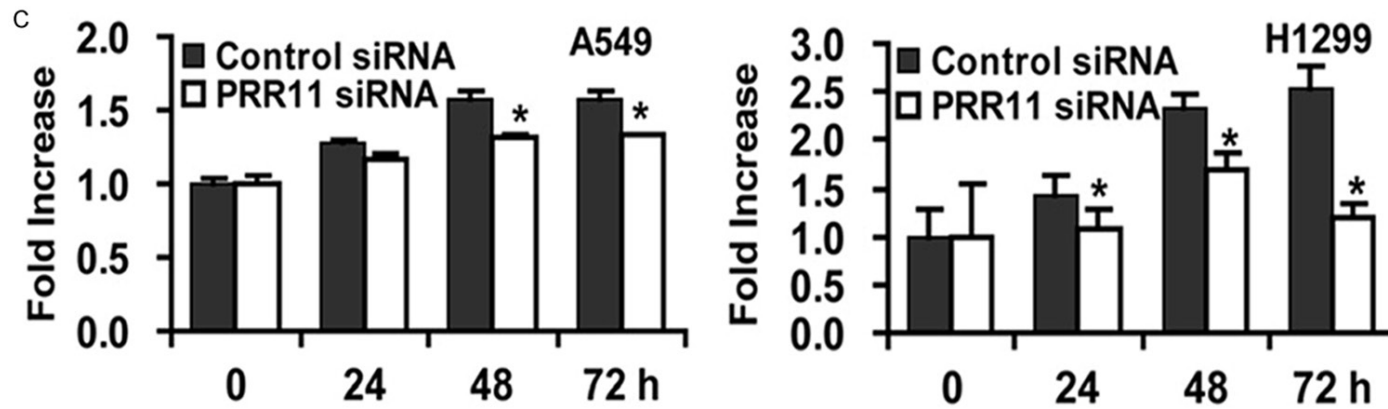


Figure 2. PRR11 depletion affects cell cycle progression and inhibits proliferation in lung cancer cells. (A) siRNA-mediated PRR11 depletion. A549 and H1299 cells were transfected with the control siRNA or PRR11 siRNA, respectively. Four-eight hours after transfection, total RNA and whole cell lysates were prepared and subjected to qRT-PCR and immunoblotting, respectively. (B) Cell cycle analysis after PRR11 depletion and (C) PRR11 depletion reduced cell proliferation. Following siRNA transfection, cells were subjected to proliferation assay at the indicated times as described. * $P < 0.05$.

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into the indicated cells using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. Cells were collected and subjected to subsequent analysis 24-72 h after transfection.

Quantitative real-time PCR

Total RNA was extracted from cultured cells using EZNA Total Rna Kit (OMEGA Bio-tek, USA), and cDNA was generated using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Otsu, Japan). Quantitative real-time PCR was performed using the SYBR Premix ExTaq II (TLiRNaseH Plus) (TaKaRa, Otsu, Japan) with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA).

Immunoblotting analysis

Cells were lysed with RIPA buffer (Beyotime, China) and boiled for 5 minutes. The protein concentration of each lysate was measured using the BCA method (Beyotime, China). Equal quantities of protein from each cell lysate were separated on SDS-polyacrylamide electrophoresis gels and transferred to PVDF membranes (Millipore, Billerica, MA). The membranes were blocked with 5% skim milk, incubated with each primary antibody overnight at 4°C, washed with TBST buffer (10 mM Tris-HCl pH7.4, 150 mM NaCl, 0.05% Tween 20) and incubated with secondary antibodies. The proteins were visualized using enhanced chemiluminescence (GE Healthcare Biosciences)

Cell growth and viability assay

Trypan blue exclusion assay was used to determine the effects of PRR11 depletion on the growth and viability of esophageal cancer cells. Briefly, 24-72 h after siRNA transfection, both floating and attached cells were collected and resuspended in PBS solution. An aliquot of cell suspension was mixed with Trypan blue solution (0.4% in PBS; Invitrogrn), and the number of cells (viable cells were unstained, nonviable cells blue) was counted under a microscope.

Statistical analyses

All statistical analyses were carried out using the SPSS 17.0 statistical software package (SPSS Inc, USA). Two-sided independent Student's *t* test was performed to analyze the significance of the relationship between PRR11 expression level and clinic-pathologic charac-

teristics. One-Way ANOVA was used to analyze the differences of the three duplicate experiments group. Overall survival curves were plotted by the Kaplan-Meier method and compared by log-rank test.

Results

PRR11 is over-expressed in lung cancer patients and metastatic lung cancer cells

To demonstrated that PRR11 was also significantly up-regulated in lung cancer samples compared with normal adjunct tissues. Quantitative RT-PCR demonstrated that, PRR11 was significantly upregulated at mRNA level in lung cancer samples compared with normal lung tissues (**Figure 1A**). Notably, overexpression of PRR11 mRNA was significantly associated with tumor grades and stages (**Figure 1A**). Consistently, Immunohistochemistry (IHC) analysis of lung cancer tissue microarray further revealed that PRR11 was also significantly upregulated in lung cancer samples compared with normal adjunct tissues. As shown in **Figure 1B**, PRR11 was mainly expressed in the cytoplasm of tumor cells but not in normal lung epithelia. Strong staining of PRR11 protein was detected in 160 (47.9%) of 231 tumors especially in squamous cell carcinoma (**Figure 1B**).

PRR11 depletion inhibits cell proliferation and affects cell cycle progression in lung cancer cells

To determine the functional involvement of PRR11 in lung cancer, the PRR11 expression was depleted via siRNA-mediated silencing in two different lung cancer cell lines, H1299 and A549, and the cell cycle profile and cellular proliferation were subsequently analyzed. As shown in **Figure 2A**, qRT-PCR and Western blot analysis demonstrated that the PRR11 expression was significantly inhibited at both mRNA and protein levels in two cell lines. Cell cycle analysis revealed that silencing PRR11 expression resulted into a remarkable S phase arrest and a mild G2/M arrest in H1299, and moderate S phase arrest in A549 (**Figure 2B**). Consequently, PRR11 depletion also caused a significant growth retardation from an earlier time point (24 h) in H1299 and later time point (48 h and 72 h) in A549, whereas control siRNA did not affect the proliferation of the cells (**Figure 2C**). These data confirmed that PRR11 was a

Depletion of PRR11 inhibits lung cancer growth

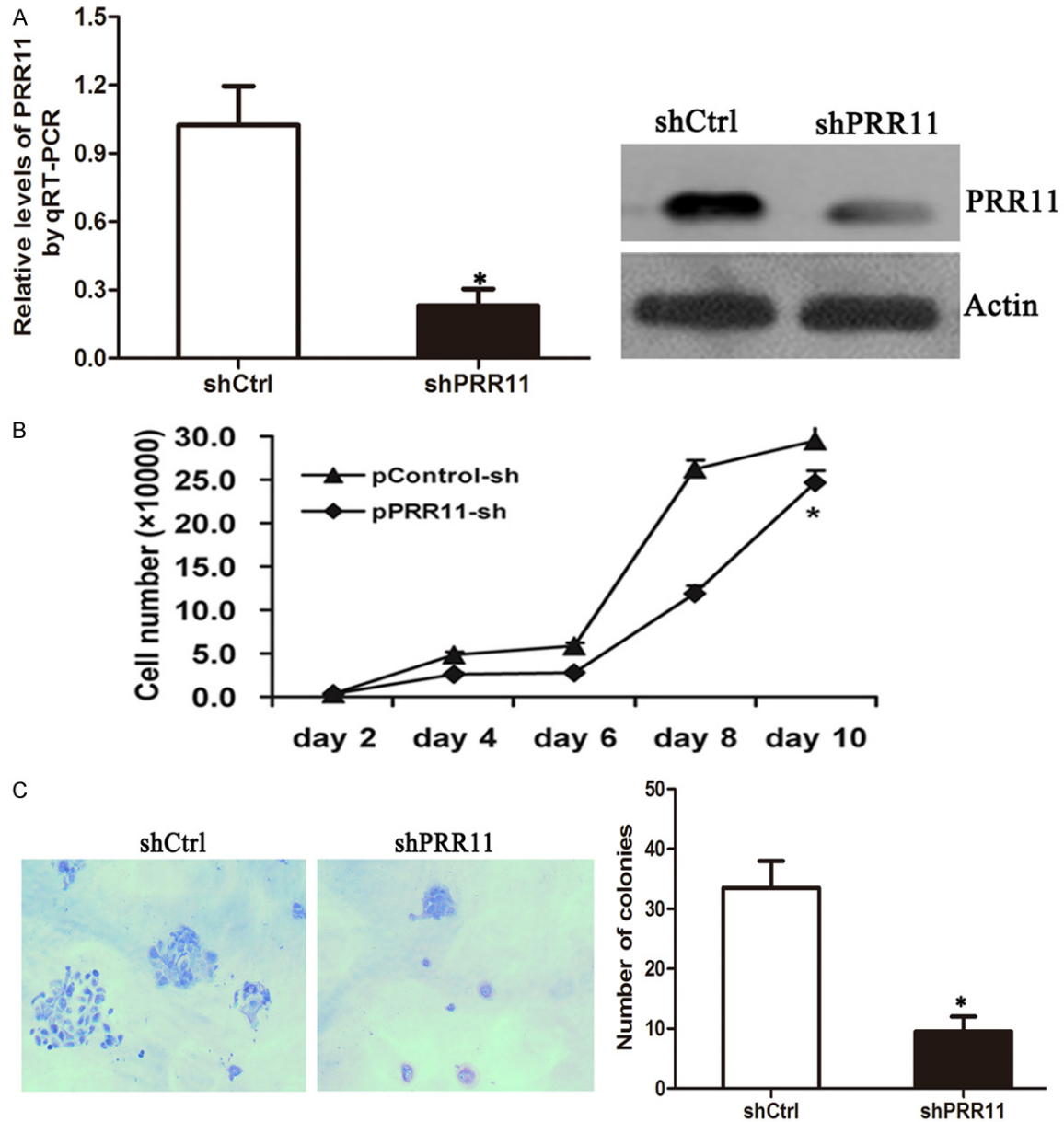


Figure 3. PRR11 depletion inhibits lung tumorigenicity in vitro. A. shRNA-mediated PRR11 depletion. A549 cells were transfected with the control shRNA or PRR11 shRNA, respectively. Stably infected colonies were selected by growth in blasticidin for 14 days, total RNA and whole cell lysates were prepared and subjected to qRT-PCR and immunoblotting. B. Cell growth assay. PRR11 stable knockdown A549 cells (Prr11-sh) and the negative control stable cells (pControl-sh) were seeded on culture plates and counted at the indicated times. C. Colony formation assay for PRR11 knockdown cells and control cells. (* $P < 0.05$).

novel cell cycle gene regulating cell cycle progression especially S phase progression.

PRR11 depletion inhibits lung tumorigenesis in vitro

Next, to demonstrate the role of PRR11 in the tumor genesis of lung cancer, we used A549 to establish a PRR11 depletion stable cell lines.

QRT-PCR and western blot analysis demonstrated the PRR11 expression was significantly depleted at both mRNA and protein levels in the stable PRR11 knockdown cells (**Figure 3A**). Similar to the transient knockdown of PRR11 in A549 cells, stable knockdown of PRR11 in these cells also resulted into a significant decrease in cell proliferation as measured by direct cell counting (**Figure 3B**). As shown in

Figure 3C, colony formation assay demonstrated that the stable PRR11 knockdown cells showed a significantly reduced colony formation activity in both number and size, as compared with the control cells.

Discussion

Cancer is essentially a genetic disease. During the multistep process of carcinogenesis, frequent genetic alterations influence key cellular pathways, and eventually lead to unrestrained cell growth and malignant phenotype [15-17]. Notably, the inactivation of tumor suppressor genes and the activation of oncogenes are the most common genetic events in cancer. Thus, a central aim of cancer research has been to identify and characterize novel genes implicated in carcinogenesis. Although a sequential series of human cancer-associated genes have been discovered over the past several decades, many more unknown cancer-associated genes remain to be identified, especially for lung cancer, the leading cause of cancer deaths in both women and men throughout the world [18, 19].

In the present study, we isolated and identified a novel gene, proline-rich protein 11 (PRR11), implicated in both cell cycle progression and lung cancer. This *in vitro* and *in vivo* study was an effort to investigate whether PRR11 could be exploited as a novel therapeutic target for the treatment of lung cancer [20]. We have found that the specific shRNA-mediated depletion of PRR11 leads to a significant decrease in cell viability, mitotic arrest followed by massive apoptosis, tumor formation in human lung cancer cell studied. These results strongly demonstrated that, apart from being of diagnostic value in esophageal cancer, inhibition of PRR11 in lung cancer may additionally serve to be of therapeutic value.

In summary, our present study strongly indicates that the specific shRNA-mediated silencing of PRR11 resulted in the elimination of lung cancer cells via the inactivation of p21/cyclinB1-mediated mitotic cell cycle arrest followed by massive apoptotic cell death. Therefore, PRR11 may serve as a potential target in the treatment of human lung cancer. Consequently and conceivably, gene therapeutic approaches and/or pharmacological small molecule inhibitors aimed at PRR11 may be developed for the management of lung cancer.

Disclosure of conflict of interest

None.

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References

- [1] Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; 127: 2893-2917.
- [2] Fossella F, Pereira JR, von Pawel J, Pluzanska A, Gorbounova V, Kaukel E, Mattson KV, Ramlau R, Szczesna A, Fidijs P, Millward M and Belani CP. Randomized, multinational, phase III study of docetaxel plus platinum combinations versus vinorelbine plus cisplatin for advanced non-small-cell lung cancer: the TAX 326 study group. *J Clin Oncol* 2003; 21: 3016-3024.
- [3] Cao JY, Liu L, Chen SP, Zhang X, Mi YJ, Liu ZG, Li MZ, Zhang H, Qian CN, Shao JY, Fu LW, Xia YF and Zeng MS. Prognostic significance and therapeutic implications of centromere protein F expression in human nasopharyngeal carcinoma. *Mol Cancer* 2010; 9: 237.
- [4] Liu AW, Cai J, Zhao XL, Jiang TH, He TF, Fu HQ, Zhu MH and Zhang SH. ShRNA-targeted MAP4K4 inhibits hepatocellular carcinoma growth. *Clin Cancer Res* 2011; 17: 710-720.
- [5] Mao N, He G, Rao J and Lv L. [Effect of silencing Bmi-1 expression in reversing cisplatin resistance in lung cancer cells and its mechanism]. *Nan Fang Yi Ke Da Xue Xue Bao* 2014; 34: 1000-1004.
- [6] Chen YY and Xu GB. Effect of circulating tumor cells combined with negative enrichment and CD45-FISH identification in diagnosis, therapy monitoring and prognosis of primary lung cancer. *Med Oncol* 2014; 31: 240.
- [7] Yan D, Zheng X, Tu L, Jia J, Li Q, Cheng L and Wang X. Knockdown of Merm1/Wbscr22 attenuates sensitivity of H460 nonsmall cell lung cancer cells to SN38 and 5FU without alteration to p53 expression levels. *Mol Med Rep* 2015; 11: 295-302.
- [8] Kim BG, Kwon HY, Sohn EJ, Hwang S, Kwon OS and Kim SH. Activation of caspases and inhibition of ribosome biogenesis mediate antitumor activity of Chijongdan in A549 non-small lung cancer cells. *BMC Complement Altern Med* 2014; 14: 420.
- [9] Shang L, Zhao J, Wang W, Xiao W, Li J, Li X, Song W, Liu J, Wen F and Yue C. [Inhibitory

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- Effect of Endostar on Lymphangiogenesis in Non-small Cell Lung Cancer and Its Effect on Circulating Tumor Cells]. *Zhongguo Fei Ai Za Zhi* 2014; 17: 722-729.
- [10] Kim MO, Lee MH, Oi N, Kim SH, Bae KB, Huang Z, Kim DJ, Reddy K, Lee SY, Park SJ, Kim JY, Xie H, Kundu JK, Ryoo ZY, Bode AM, Surh YJ and Dong Z. [6]-shogaol inhibits growth and induces apoptosis of non-small cell lung cancer cells by directly regulating Akt1/2. *Carcinogenesis* 2014; 35: 683-691.
- [11] Roche J, Nasarre P, Gemmill R, Baldys A, Pontis J, Korch C, Guilhot J, Ait-Si-Ali S and Drabkin H. Global Decrease of Histone H3K27 Acetylation in ZEB1-Induced Epithelial to Mesenchymal Transition in Lung Cancer Cells. *Cancers (Basel)* 2013; 5: 334-356.
- [12] Hsieh YS, Chu SC, Hsu LS, Chen KS, Lai MT, Yeh CH and Chen PN. *Rubus idaeus* L. reverses epithelial-to-mesenchymal transition and suppresses cell invasion and protease activities by targeting ERK1/2 and FAK pathways in human lung cancer cells. *Food Chem Toxicol* 2013; 62: 908-918.
- [13] Li J, Shi SB, Shi WL, Wang Y, Yu LC, Zhu LR and Ge LP. LUNX mRNA-positive cells at different time points predict prognosis in patients with surgically resected nonsmall cell lung cancer. *Transl Res* 2014; 163: 27-35.
- [14] Chen Q, Jiao D, Hu H, Song J, Yan J, Wu L and Xu LQ. Downregulation of LIMK1 level inhibits migration of lung cancer cells and enhances sensitivity to chemotherapy drugs. *Oncol Res* 2013; 20: 491-498.
- [15] Li H, Song F, Chen X, Li Y, Fan J and Wu X. Bmi-1 regulates epithelial-to-mesenchymal transition to promote migration and invasion of breast cancer cells. *Int J Clin Exp Pathol* 2014; 7: 3057-3064.
- [16] Li H, Chen X, Gao Y, Wu J, Zeng F and Song F. XBP1 induces snail expression to promote epithelial-to-mesenchymal transition and invasion of breast cancer cells. *Cellular Signalling* 2015; 27: 82-89.
- [17] Carter SL, Eklund AC, Kohane IS, Harris LN and Szallasi Z. A signature of chromosomal instability inferred from gene expression profiles predicts clinical outcome in multiple human cancers. *Nat Genet* 2006; 38: 1043-1048.
- [18] Wikman H and Kettunen E. Regulation of the G1/S phase of the cell cycle and alterations in the RB pathway in human lung cancer. *Expert Rev Anticancer Ther* 2006; 6: 515-530.
- [19] Gugger M, Kappeler A, Vonlanthen S, Altermatt HJ, Ris HB, Lardinois D, Borner MM, Heighway J and Betticher DC. Alterations of cell cycle regulators are less frequent in advanced non-small cell lung cancer than in resectable tumours. *Lung Cancer* 2001; 33: 229-239.
- [20] Vincenzi B, Schiavon G, Silletta M, Santini D, Perrone G, Di Marino M, Angeletti S, Baldi A and Tonini G. Cell cycle alterations and lung cancer. *Histol Histopathol* 2006; 21: 423-435.