

Original Article

The role of PRRX1 in the apoptosis of A549 cells induced by cisplatin

Hongbin Zhu¹, Gengyun Sun², Jiahui Dong², Liming Fei²

¹Department of Respiratory Medicine, The First Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui, China; Chaohu Hospital of Anhui Medical University, Chaohu 238000, Anhui, China; ²Department of Respiratory Medicine, The First Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui, China

Received May 26, 2016; Accepted January 12, 2017; Epub February 15, 2017; Published February 28, 2017

Abstract: Paired related homeobox1 (PRRX1) was a newly identified Epithelial mesenchymal transition (EMT) inducer. It was found that the decreased expression of PRRX1 in breast cancer and liver cancer could enable tumor cells to obtain tumor stem cell characteristics in vitro studies. However, the role of PRRX1 in lung cancer was still unknown. The down-regulated PRRX1 gene in A549 cells was established by slow virus infection in this study. The apoptosis of A549 cells was observed after the treatment of different concentrations of cisplatin and the role of PRRX1 in the apoptosis of A549 cells was explored. MTT results showed that down-regulated PRRX1 gene could resist the inhibitory effect of cisplatin on cell proliferation. The results of flow cytometry assay showed that down-regulated PRRX1 gene could reduce the apoptosis and promote A549 cells to enter G2 phase. Mitochondrial membrane potential detection showed that PRRX1 gene could inhibit the decrease of mitochondrial membrane potential. Western blotting results showed that down-regulated PRRX1 gene could reduce the expression levels of Caspase3, caspase9, Apaf-1 and cytochrome C. In a word, down-regulation of PRRX1 could cause lung cancer cells to produce anti apoptotic ability and resistance to cisplatin, which maybe through caspase3 pathway.

Keywords: PRRX1, A549 cells, cisplatin, apoptosis

Introduction

Lung cancer is thought to be the leading cause of cancer death in the world [1]. Chemotherapy is the main method for the treatment of inoperable lung cancer. However, even the latest chemotherapy drugs are not satisfactory [2]. Drug resistance is the main obstacle to improve the efficacy of chemotherapy drugs and the survival rate of patients.

Carcinoma cells can undergo a phenotypic transformation, epithelial mesenchymal transition (EMT) enables them to invade, intravasate and navigate through a network of thin vessels while acquiring stem cell-like properties [3-5]. It was also observed in both adenocarcinoma and squamous cell carcinoma, and it was related to the proliferation and drug resistance of tumor cells [6-9]. Paired related homeobox1 (PRRX1) was a newly identified EMT inducer and was different from the classic EMT inducers. It was found that the decreased express-

ion of PRRX1 in breast cancer and liver cancer could enable tumor cells to obtain tumor stem cell characteristics in vitro studies, and there was a significant correlation between the abnormal expression of PRRX1 and the poor prognosis of breast cancer, lung squamous cell carcinoma and colorectal cancer [10, 11]. However, the role of PRRX1 in lung cancer was still unknown, especially in relation to drug resistance.

In this study, we explored the correlation between the abnormal expression of PRRX1 and the drug resistance of lung cancer.

Materials and methods

Cell culture and transfection

Human lung cancer cell line A549 was obtained from Elemental Laboratory of Anhui Medical University. A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)

PRRX1 and the apoptosis of A549 cells

Table 1. Primers used in this study

Gene	Sequence	Use	Size
PRRX1	TGATGCTTTTGTGCGAGAAGA	forward primer	135 bp
	AGGAAGCGTTTTTATTGGCT	reverse primer	
GAPDH	TGTGGGCATCAATGGATTGG	forward primer	116 bp
	ACACCATGTATTCCGGGTCAAT	reverse primer	

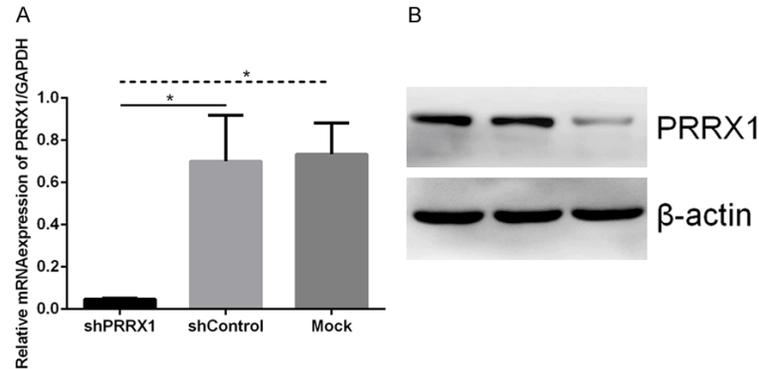


Figure 1. PRRX1 gene was silenced successfully. A: RT-PCR results (* $P < 0.05$); B: Western blotting results. It showed that the expression levels of PRRX1 in shPRRX1 group were significantly lower than that of Control group and shmock group.

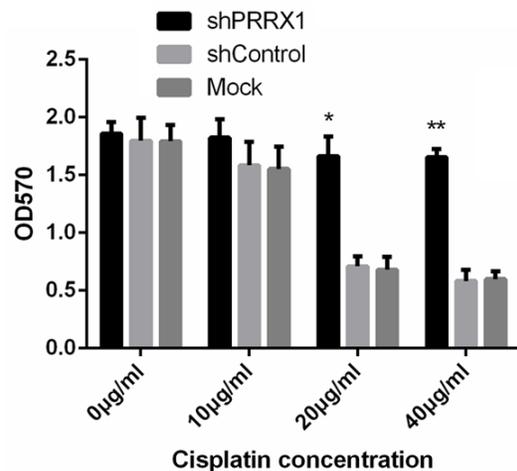


Figure 2. Comparison of cell proliferation in different groups after the treatment of different concentrations of cisplatin. The cell proliferation was significantly inhibited in the control group and non-infected group following the cisplatin concentration increased. However, in the slow virus infection group, the cell proliferation was not significantly inhibited.

medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (GIBCO) at

37°C in 5% CO₂. They were divided into PRRX1-siRNA group and control group. The concentration of PAC-1 is 50 µg/ml. Construction of lentiviral vector for PRRX1 RNA interference and package the virus were completed by the Shanghai Ji Kai gene Chemical Technology Co., Ltd.. A549 cells were transfected using lipofectamine 2000 (Life Technologies) according to the manual.

RNA extraction and real-time PCR

Total RNA was extracted using RNAiso Plus Kit (Takara, Dalian, China) according to the manufacturer's protocol. Their concentration and purity were detected with Agilent 2100 Bioanalyzer. 1 µg RNA was subjected to reverse transcription using PrimeScript™ Master Mix (Takara, Dalian, China). Real-time PCR were performed using SYBR Premix EX Taq™ II (Takara, Dalian, China). The primers used in this study were shown in **Table 1**. GAPDH gene was used as an internal control for normalization of RNA quantity and quality differences in all samples. Reaction parameters were 95°C for 45 sec, 95°C for 5 sec and 60°C for 30 sec with 40 cycles. The data were analyzed with a normalized gene expression method through the IQ5 Optical System Software.

Western blotting detection

The cells were harvested and lysed with RIPA lysis buffer and total proteins were extracted and analyzed with SDS-PAGE electrophoresis. Then it was electrotransferred to the PVDF membrane. After the transmembrane, PVDF membrane was rinsed with TBS for 10 to 15 min, placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder and shook at room temperature for one hour. It was incubated at room temperature for two hours after added with appropriate dilution degree of primary antibodies Apaf-1 (Santa Cruz Biotechnology, Inc., 1:800); Caspase 9 (Santa

PRRX1 and the apoptosis of A549 cells

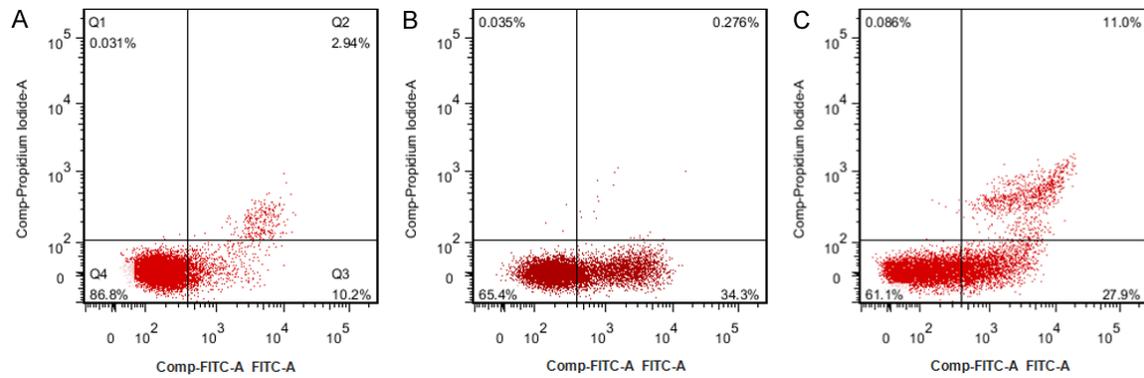


Figure 3. Apoptotic effects of PRRX1 siRNA on A549 cells treated with 20 µg/ml cisplatin. A: shPRRX1; B: shMock; C: Control. PRRX1 gene could reduce cell apoptosis. The rate of apoptotic cells was about 30% in the control group and non-infected group, while it was about 10% in infection group.

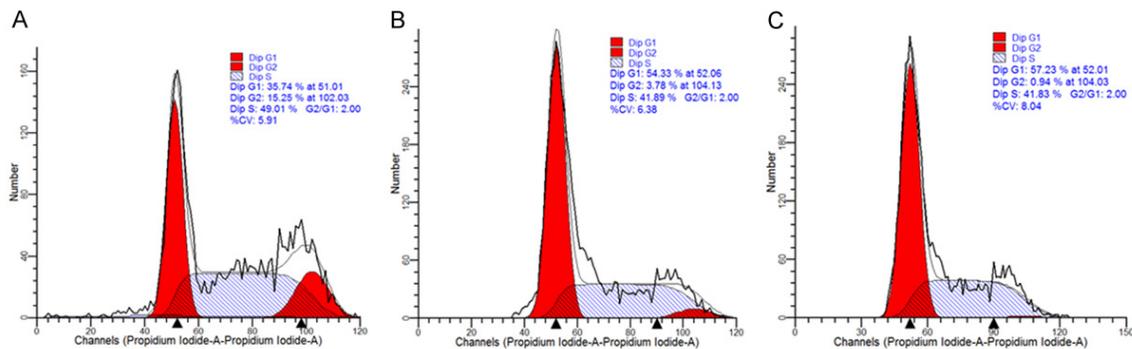


Figure 4. PRRX1 gene could promote A549 cells to enter G2 phase. A: shPRRX1; B: shMock; C: Control. It showed that G2/S phase of A549 cells significantly increased in infection group than that of other groups. PRRX1 gene could promote A549 cells to enter G2 phase.

Cruz Biotechnology, Inc., 1:800) and Cytochrome C (Santa Cruz Biotechnology, Inc., 1:800). Then the membrane was rinsed with TBST for three times (5 to 10 minutes one time). The membrane was incubated at room temperature for one hour with HRP labeled secondary antibody (1:10000) diluted with TBST containing 0.05% (w/v) skimmed milk powder and rinsed for three times with TBST (5 to 10 minutes at a time). The protein bands were scanned and quantified as a ratio to β -actin.

Proliferation ability of cells was detected by MTT method

The proliferation ability of cells was detected by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] method. The cells were divided into shPRRX1 group, sh-control group and mock group. Their densities were adjusted

to 2×10^3 cells/ml and inoculated in 96 well plates with 100 µl/well and incubated at 37°C with 5% CO₂. The cells were treated with 5 µg/ml, 10 µg/ml, 20 µg/ml and 40 µg/ml cisplatin respectively and cultured for 48 h. Then 10 µl MTT (5 mg/ml, Roche Applied Science, Basel, Switzerland) was added into each wells and cultured for 4 h. DMSO (150 µl) was added and oscillated at low speed for 10 min. The absorbance value of each well was measured at 490 nm wavelength using the enzyme linked immunosorbent assay.

Cell cycle and apoptosis detection

The cell cycle was detected by BD Biosciences FACSCalibur Flow Cytometry System (BD Biosciences, NJ, USA). The cell density was inoculated in 6 cm dish and used for detection when they reached to 85% fusion. Fluorescein

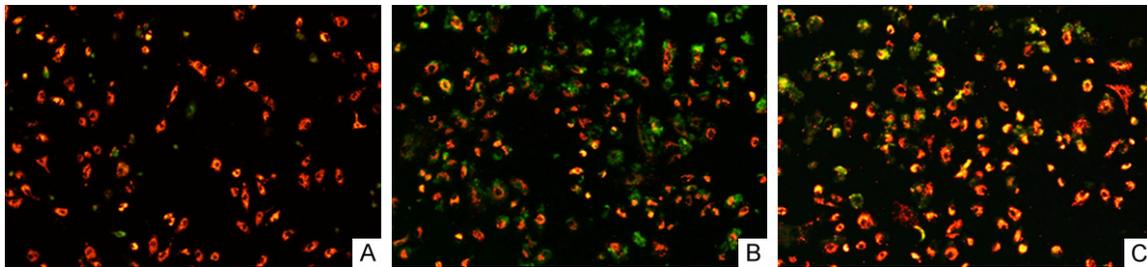


Figure 5. PRRX1 siRNA inhibited apoptosis induced by cisplatin through reduction of mitochondrial membrane potential. A: shPRRX1; B: shMock; C: Control. The mitochondrial membrane potential in infection group was lower than that of other groups. PRRX1 siRNA inhibited apoptosis induced by cisplatin through reduction of mitochondrial membrane potential.

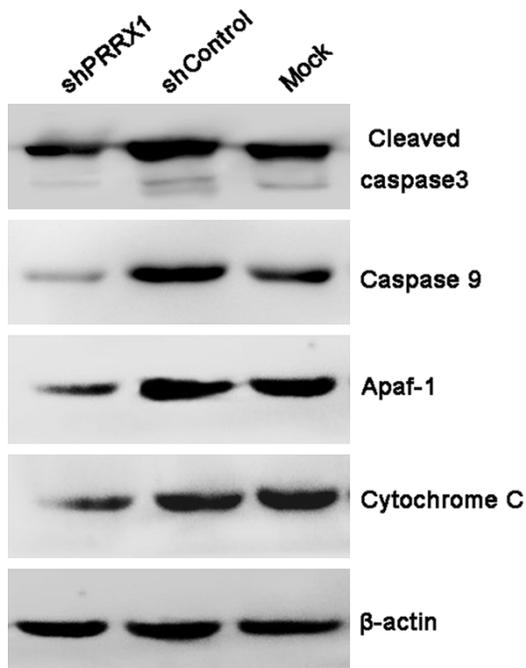


Figure 6. PRRX1 siRNA could down-regulate the expression of caspase-3 and inhibit the release of cytochrome C from mitochondria. Western blotting results showed that the expression levels of caspase-3, cytochrome C and Apaf-1 in infection group were lower than that of other groups. So PRRX1 siRNA could down-regulate the expression of caspase-3 and inhibit the release of cytochrome C from mitochondria.

Annexin V-FITC/PI double labeling was performed with the Annexin V-FITC Apoptosis Detection Kit (Beckman) to detect the apoptotic effects of PRRX1 on A549 cells. The cells were stained with Annexin V-FITC and PI according to the instructions of the kit manual. The apoptotic cells were determined with a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo V7 software (Tree Star, Oregon, USA).

Determination of mitochondrial membrane potential

The mitochondrial membrane potential was determined using JC-1 Mitochondrial Membrane Potential Detection Kit (Beyotime Inst) according to the manual. The cells were treated with 2 $\mu\text{g/ml}$ JC-1 and cultured for 20 min at 37°C. Cells with red and green fluorescence were observed under a fluorescence microscope and counted.

Statistical analysis

The results are expressed as mean \pm SD, and the Mann-Whitney U test was used to evaluate the differences between groups. A value of $P < 0.05$ and $P < 0.01$ was taken to denote statistical significance.

Results

PRRX1 gene was silenced successfully

RT-PCR results showed that the expression levels of PRRX1 in shPRRX1 group were significantly lower than that of control group and shmock group (**Figure 1A**) and western blotting results also showed the similar results (**Figure 1B**), which suggested that down regulation of PRRX1 gene was established successfully.

PRRX1 gene could reduce cell apoptosis

The proliferation ability of cells was detected by MTT method. The results showed that the cell proliferation was significantly inhibited in the control group and non-infected group following the cisplatin concentration increased, and it reached the best results at a concentration of 20 $\mu\text{g/ml}$. However, in the slow virus infection group, the cell proliferation was not significantly inhibited even when the concentration of cisplatin was 40 $\mu\text{g/ml}$ (**Figure 2**).

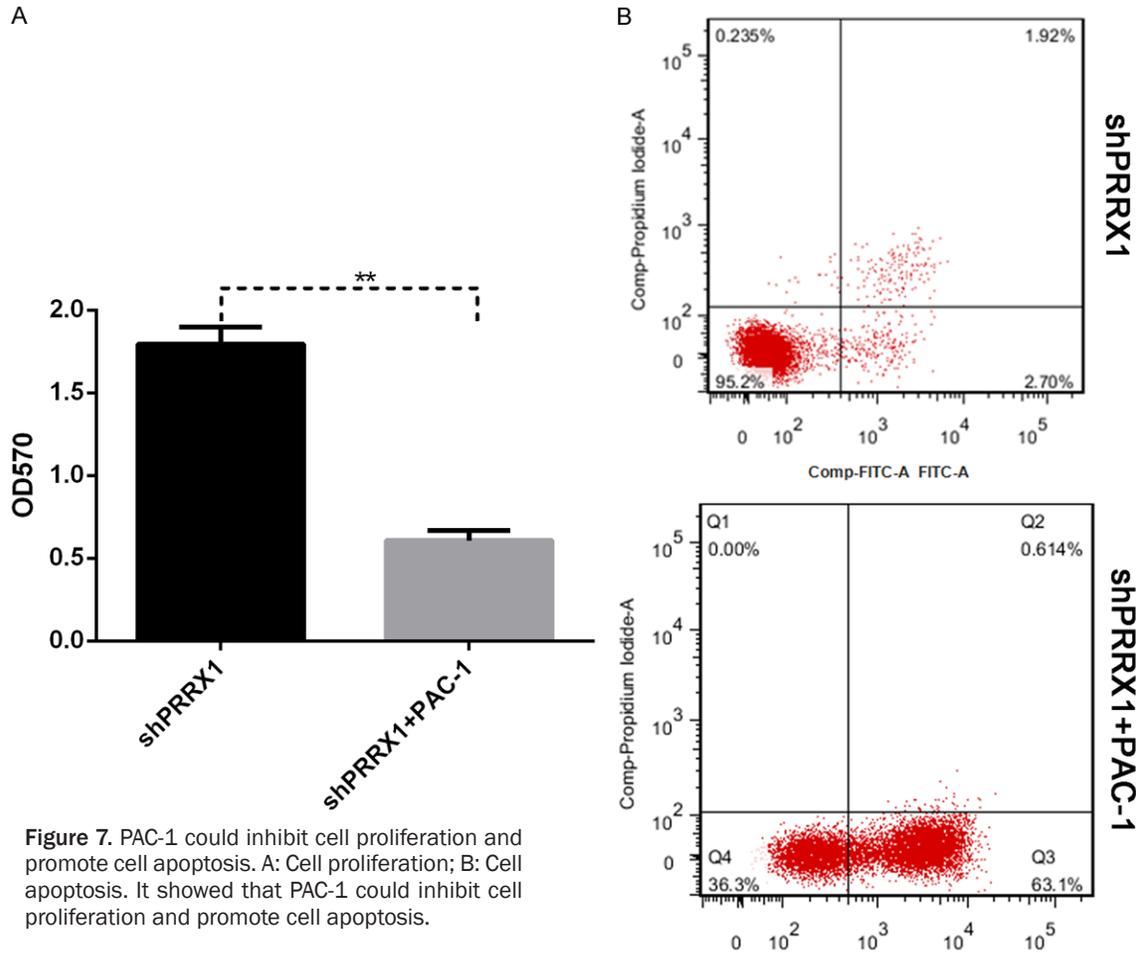


Figure 7. PAC-1 could inhibit cell proliferation and promote cell apoptosis. A: Cell proliferation; B: Cell apoptosis. It showed that PAC-1 could inhibit cell proliferation and promote cell apoptosis.

Apoptotic analysis showed that the rate of apoptotic cells was about 30% in the control group and non-infected group, while it was about 10% in infection group (Figure 3), which suggested that PRRX1 gene could reduce cell apoptosis.

The flow cytometry results were shown in Figure 4. It showed that G2/S phase of A549 cells significantly increased in infection group than that of other groups. So PRRX1 gene could promote A549 cells to enter G2 phase.

PRRX1 siRNA inhibited apoptosis induced by cisplatin through reduction of mitochondrial membrane potential

The results of mitochondrial membrane potential were shown in Figure 5. It showed that the mitochondrial membrane potential in infection group was lower than that of other groups, which suggested that PRRX1 siRNA inhibited

apoptosis induced by cisplatin through reduction of mitochondrial membrane potential.

PRRX1 siRNA could down regulate the expression of caspase-3

The expression levels of caspase-3, cytochrome C and Apaf-1 were detected by Western blotting method. The results showed that their expression levels in infection group were lower than that of other groups, which suggested that PRRX1 siRNA could down regulate the expression of caspase-3 and inhibit the release of cytochrome C from mitochondria (Figure 6). When PAC-1 was added into medium, it could promote the apoptosis (Figure 7).

Statistical analysis

Statistical analysis was performed by SPSS 16.0 software using t-test. A value of $P < 0.05$

and $P < 0.01$ was taken to denote statistical significance.

Discussion

Enhancing the efficiency of chemotherapy drugs to inhibit the anti apoptotic pathway or promoting apoptosis is the main way to improve the treatment of lung cancer.

PRRX1 is a transcriptional co-activator and can improve the DNA binding activity of serum response factor [12]. Previous study showed that PRRX1 could promote EMT by regulating E-cadherin, N-cadherin and vimentin [13, 14]. In this study, we found that down regulation of the PRRX1 expression of in A549 cells could produce anti-apoptotic effects for resistance to apoptosis induced by cisplatin, the anti apoptotic mechanism may be by inhibiting the cell to produce Caspase3.

The correlation between abnormal expression of PRRX1 gene and lung cancer drug resistance has not been reported. However, it has been reported that the abnormal expression of PRRX1 gene is significantly associated with poor prognosis in other cancers. Ocan^ˆa found that abnormal expression of PRRX1 made cancer cells obtain cancer stem cell like properties via MET pathway, and was associated with the metastatic colonization and poor prognosis in breast and lung squamous cell carcinoma [10]. Guo found that PRRX1 promotes EMT in gastric cancer cells through the activation of Wnt/ β -catenin signaling and that PRRX1 upregulation is closely correlated with gastric cancer metastasis [15]. Hidenari found that abnormal expression of PRRX1 made hepatocellular carcinoma cells obtain cancer stem cell like properties and was associated with poor prognosis [16]. Takano found that Prrx1b promotes invasion, tumor dedifferentiation, and EMT. In contrast, Prrx1a stimulates metastatic outgrowth in the liver, tumor differentiation, and MET [17]. In addition, it was also found that the abnormal expression of PRRX1 gene can produce radiation resistance in hepatocellular carcinoma cells [18-20]. However, there was no report about the abnormal expression of PRRX1 gene and chemotherapy resistance. In this study, we found that downregulation of PRRX1 gene expression could cause A549 cells to produce resistance to cisplatin. We speculated that the role of PRRX1 gene in tumor metastasis and

progression is different in different types of tumors.

In a word, in this study we found that self-regulation of PRRX1 expression could cause lung cancer cells to produce anti apoptotic ability and resistance to cisplatin, which maybe through caspase3 pathway. PRRX1 could be a new molecular marker for lung cancer chemotherapy.

Disclosure of conflict of interest

None.

Address correspondence to: Gengyun Sun, Department of Respiratory Medicine, The First Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui, China. Tel: 86-13966673211; Fax: 86-551-63633742; E-mail: sungengyun1@sina.com

References

- [1] Jemal A, Siegel R, Xu J and Ward E. Cancer statistics, 2010. *CA Cancer J Clin* 2010; 60: 277-300.
- [2] Rigas JR. Do newer chemotherapeutic agents improve survival in non-small cell lung cancer? *Semin Oncol* 1998; 25: 5-9.
- [3] Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Briskin C, Yang J and Weinberg RA. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 2008; 133: 704-715.
- [4] Morel AP, Lièvre M, Thomas C, Hinkal G, Ansieau S, Puisieux A. Generation of breast cancer stem cells through epithelial-mesenchymal transition. *PLoS One* 2008; 3: e2888.
- [5] Nieto MA. The ins and outs of the epithelial to mesenchymal transition in health and disease. *Annu Rev Cell Dev Biol* 2011; 27: 347-376.
- [6] Haraguchi N, Ishii H, Mimori K, Tanaka F, Ohkuma M, Kim HM, Akita H, Takiuchi D, Hatano H, Nagano H, Barnard GF, Doki Y and Mori M. CD13 is a therapeutic target in human liver cancer stem cells. *J Clin Invest* 2010; 120: 3326-3339.
- [7] Yamashita T, Ji J, Budhu A, Forgues M, Yang W, Wang HY, Jia H, Ye Q, Qin LX, Wauthier E, Reid LM, Minato H, Honda M, Kaneko S, Tang ZY and Wang XW. EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. *Gastroenterology* 2009; 136: 1012-1024.
- [8] Ma S, Lee TK, Zheng BJ, Chan KW and Guan XY. CD133+ HCC cancer stem cells confer chemoresistance by preferential expression of the

PRRX1 and the apoptosis of A549 cells

- Akt/PKB survival pathway. *Oncogene* 2008; 27: 1749-1758.
- [9] Nieto MA and Cano A. The epithelial-mesenchymal transition under control: Global programs to regulate epithelial plasticity. *Semin Cancer Biol* 2012; 22: 361-368.
- [10] Ocaña OH, Corcoles R, Fabra A, Moreno-Bueno G, Acloque H, Vega S, Barrallo-Gimeno A, Cano A and Nieto MA. Metastatic colonization requires the repression of the epithelial-mesenchymal transition inducer *Prrx1*. *Cancer Cell* 2012; 22: 709-724.
- [11] Takahashi Y, Sawada G, Kurashige J, Uchi R, Matsumura T, Ueo H, Takano Y, Akiyoshi S, Eguchi H, Sudo T, Sugimachi K, Doki Y, Mori M and Mimori K. Paired related homoeobox 1, a new EMT inducer, is involved in metastasis and poor prognosis in colorectal cancer. *Br J Cancer* 2013; 109: 307-311.
- [12] Grueneberg DA, Natesan S, Alexandre C and Gilman MZ. Human and *Drosophila* homeodomain proteins that enhance the DNA-binding activity of serum response factor. *Science* 1992; 257: 1089-1095.
- [13] Reichert M, Takano S, von Burstin J, Kim SB, Lee JS, Ihida-Stansbury K, Hahn C, Heeg S, Schneider G, Rhim AD, Stanger BZ and Rustgi AK. The *Prrx1* homeodomain transcription factor plays a central role in pancreatic regeneration and carcinogenesis. *Genes Dev* 2013; 27: 288-300.
- [14] Greer RL, Staley BK, Liou A and Hebrok M. *Numb* regulates acinar cell dedifferentiation and survival during pancreatic damage and acinar-to-ductal metaplasia. *Gastroenterology* 2013; 145: 1088-1097.
- [15] Guo J, Fu Z, Wei J, Lu W, Feng J and Zhang S. *PRRX1* promotes epithelial-mesenchymal transition through the Wnt/ β -catenin pathway in gastric cancer. *Med Oncol* 2015; 32: 393.
- [16] Hirata H, Sugimachi K, Takahashi Y, Ueda M, Sakimura S, Uchi R, Kurashige J, Takano Y, Nanbara S, Komatsu H, Saito T, Shinden Y, Iguchi T, Eguchi H, Atsumi K, Sakamoto K, Doi T, Hirakawa M, Honda H and Mimori K. Downregulation of *PRRX1* confers cancer stem cell-like properties and predicts poor prognosis in hepatocellular carcinoma. *Ann Surg Oncol* 2015; 22 Suppl 3: S1402-1409.
- [17] Takano S, Reichert M, Bakir B, Das KK, Nishida T, Miyazaki M, Heeg S, Collins MA, Marchand B, Hicks PD, Maitra A and Rustgi AK. *Prrx1* isoform switching regulates pancreatic cancer invasion and metastatic colonization. *Genes Dev* 2016; 30: 233-247.
- [18] Zhang Y, Zheng L, Huang J, Gao F, Lin X, He L, Li D, Li Z, Ding Y and Chen L. *MiR-124* radiosensitizes human colorectal cancer cells by targeting *PRRX1*. *PLoS One* 2014; 9: e93917.
- [19] Chen Y, Yu D, Zhang H, He H, Zhang C, Zhao W and Shao RG. *CD133(+)**EpCAM(+)* phenotype possesses more characteristics of tumor initiating cells in hepatocellular carcinoma Huh7 cells. *Int J Biol Sci* 2012; 8: 992-1004.
- [20] Yamada T, Abei M, Danjoh I, Shiota R, Yamashita T, Hyodo I and Nakamura Y. Identification of a unique hepatocellular carcinoma line, Li-7, with *CD13(+)* cancer stem cells hierarchy and population change upon its differentiation during culture and effects of sorafenib. *BMC Cancer* 2015; 15: 260.