JBUON 2015; 20(1): 173-179
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

miR-150 is a factor of survival in prostate cancer patients

Liu Dezhong[1], Zhang Xiaoyi[1], Long Xianlian[2], Zhang Hongyan[3], Zhao Guohua[1], Sun Bo[1], Zou Shenglei[1], Zou Lian[1]

[1]Department of Urology and [2]Department of Clinical Laboratory, Second Artillery General Hospital of PLA, Beijing, China; [3]Beijing Shouer Pharmaceutical Factory, Beijing, China

Summary

Purpose: *Prostate cancer (PC) is the most common malignant disease in males and the second leading cause of cancer related deaths in men in developed countries. The purpose of this study was to investigate whether microRNA (miR)-150 is a factor influencing survival in prostate cancer patients.*

Methods: *miR-150 mRNA and protein expression levels in prostatic cancer cell lines and healthy tissues were determined by quantitative (q) RT-PCR and Western blotting. Additionally, the protein expression of miR-150 was detected by immunohistochemistry.*

Results: *High miR-150 expression was positively correlated with tumor recurrence or metastasis (p=0.010). In addition, PC patients with high miR-150 expression had significantly poorer overall survival/OR (hazard ratio/HR, 1.87; 95% confidence interval/CI, 1.19-2.94; p=0.006) and poorer disease-free survival/DFS (HR, 1.90; 95% CI, 1.21-2.98; p=0.005) than those with low miR-150 expression. The cumulative 5-year OS was only 35.19% (95% CI, 26.18-44.20) in the high miR-150 expression group, whereas it was 55.93% (95% CI, 43.26-68.60) in the low miR-150 expression group (p<0.05). Multivariate Cox regression analysis demonstrated that the expression of miR-150, tumor size, and number of tumor lesions were independent prognostic predictors for OS in PC patients.*

Conclusion: *miR-150 was overexpressed in PC at both the mRNA and protein levels, and high expression of miR-150 could serve as a novel and reliable prognostic biomarker for PC patients.*

Key words: *biomarker, miR-150, prognosis, prostate cancer*

Introduction

PC is the most common malignant disease in males and the second leading cause of cancer related deaths in men in developed countries [1]. Although current treatment strategies based on androgen ablation can produce very positive initial results, the majority of cases relapse in <2 years with a more aggressive hormone independent form [2]. Currently, there is no curative treatment for androgen-independent PC. Development of more effective treatment strategies, particularly for androgen-independent cancer, relies on understanding further the molecular mechanisms responsible for the malignant progression. Thus, identification of cancer-related genes and understanding how these genes function inside cancer cells to promote or to suppress tumorigenesis are important initial steps for either better diagnosis or prognosis and for the identification of better therapeutic targets in the future.

Recently, miRs have attracted attention for their involvement in the regulation of gene expression. miRs are small non-coding RNAs, approximately 18-25 nucleotides in length, which partially bind to the 3'-untranslated region (3'-UTR) of target mRNAs, leading to mRNA degradation and/or translational repression [3]. Many miRs play an essential role in cellular processes, such as proliferation, differentiation, apoptosis, and cancer progression, depending on their specific gene targets. The miR-150 gene is on the chro-

Correspondence to: Liu Dezhong, MD. Department of Urology, Second Artillery General Hospital of PLA, 16th Xin Jie Kou Wai Street, Beijing 100088, China. Tel: +86 10 66343107; Fax: +86 10 51813837; E-mail: dezh_liu@163.com
Received: 22/03/2014; Accepted: 03/04/2014

mosome 19q13 and is expressed at low levels in most solid tumors [4,5]. Downregulation of miR-150 expression acts as an anti-apoptotic factor in human diffuse gastric cancer [6] and adrenocorticotropic hormone-secreting pituitary tumors [7]. A recent research has shown that miR-150 expression was significantly lower in cancer tissues and colorectal cancer compared to adjacent non-cancerous tissues [8,9]. Injection of mouse lymphoma cell lines into mice expressing miR-150 produced fewer tumor cells in vivo [10]. Taken together, these findings also support a causative role for changes in miR-150 expression during tumorigenesis. However, the expression of miR-150 in PC patients is unknown.

The purpose of this study was to clarify the clinicopathological significance of miR-150 in PC patients.

Methods

Cell lines and tissue samples

Four human prostatic cancer cell lines, including DU145, PC-3, LNCaP and PC-3M-IE8, and one immortalized normal prostatic cell line RWPE-1 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All of the cell lines were maintained in RPMI-1640 (Invitrogen Inc., Carlsbad, CA,USA) supplemented with 10% fetal bovine serum (FBS).

A total of 167 paraffin-embedded specimens from patients with PC who had undergone curative prostatectomy were obtained from the Second Artillery General Hospital of PLA between January 2004 to December 2012. In addition, 4 pairs of PC and adjacent to tumor healthy tissues to tumor were also collected from the Second Artillery General Hospital of PLA. All cases were histologically confirmed and patients did not receive any anti-tumor treatment before tumor removal. After explaining the purpose of this study, written informed consent was obtained from all patients, and the study protocol was approved by the Institutional Ethics Review Boards of both hospitals.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from the cultured PC cells and healthy tissues using TRIzol reagent (Invitrogen Life Technologies, Ontario, Canada). 2 μg total RNA of each sample was reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and random primers (Promega) for 60 min at 37 ^{O}C and 15 min at 70 ^{O}C. qRT-PCR was performed using Platinum SYBR Green qPCR SuperMix-UDG reagents (Invitrogen, Carlsbad, CA, USA) on the Bio-Rad CFX96 qRT-PCR detection system (Applied Biosystems Inc., Foster City, CA, USA). The qRT-PCR reactions were pre-incubated for 10 min at 95 ^{O}C, followed by 40 cycles of denaturation for 30 s at 95 ^{O}C and annealing for 1 min at 60 ^{O}C. Reactions containing neither reverse transcriptase nor template were used as negative controls, and all of the reactions were conducted in triplicate. Glyceraldeyde-3-phosphate dehydrogenase (GAPDH) was used as normalization control, and the relative expression level was calculated using the $2^{-\Delta\Delta CT}$ equation (Livak and Schmittgen 2001).

Western blotting

Total protein was extracted from the cultured PC cells and healthy tissues using RIPA (radio immunoprecipitation assay) buffer (Cell Signaling Technology, Danvers, MA) containing protease inhibitor (Roche Applied Science, Mannheim, Germany), and protein concentrations were determined with the Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). 40 μg total protein of each sample were separated with 9% SDS-polyacrylamide gel and transferred to PVDF (polyvinylidene fluoride) membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% fat-free milk, incubated with mouse polyclonal anti-miR-150 antibody (1:1000), and further incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:2000; Sigma, St. Louis, MO, USA). The anti-GAPDH antibody (1:1000; Abcamplc) was used as loading control, and the bands were detected using the enhanced chemiluminescence assay.

Immunohistochemistry

Immunohistochemical staining was used to detect the miR-150 protein expression in 167 paraffin-embedded PC tissue specimens. In brief, paraffin-embedded samples were cut into 5 μm sections, and sections were dried overnight at 37 ^{O}C, followed by deparaffinization with xylene and rehydration through a series of graded alcohol. The sections were treated with 3% hydrogen peroxide for 20 min to inhibit the endogenous peroxidase activity, and then microwaved for antigenic retrieval in ethylene diamine tetraacetic acid (EDTA) buffer. Non-specific antibody binding was blocked with 1% bovine serum albumin. Subsequently, the sections were incubated with mouse polyclonal anti-miR-150 antibody (1:100) overnight at 4 ^{O}C. After rinsing, the sections were incubated with a biotinylated secondary antibody bound to streptavidin-horseradish peroxidase complex. Finally, the sections were stained with 3,3-diaminobenzidine, and counterstained with hematoxylin. Negative controls were employed, in which the primary antibody was replaced by phosphate buffered saline (PBS).

The evaluation of immunohistochemical results was independently performed by three pathologists who were blinded to the clinical data. The protein expression of miR-150 was evaluated by combining the intensity of staining and the proportion of positively stained tumor cells as follows: Briefly, the intensity of

Table 1. Correlation between miR-150 expression and the clinicopathological features of PC

Characteristics	*Patients, N*	*Expression of miR-150*		*p value*
		Low, N (%)	*High, N (%)*	
Gender				
Male	139	49 (83)	90 (83)	0.963
Female	28	10 (17)	18 (17)	
Age, years				
≤50	111	41 (69)	70 (65)	0.541
>50	56	18 (31)	38 (35)	
Tumor size, cm				
≤5	42	13 (22)	29 (27)	0.493
>5	125	46 (78)	79 (73)	
Tumor lesion number				
Solitary	117	43 (73)	74 (69)	0.556
Multiple	50	16 (27)	34 (31)	
Cirrhosis				
Absent	42	12 (20)	30 (28)	0.29
Present	125	47 (80)	78 (72)	
Recurrence or metastasis				
No	71	33 (15)	38 (28)	0.01
Yes	96	26 (85)	70 (72)	

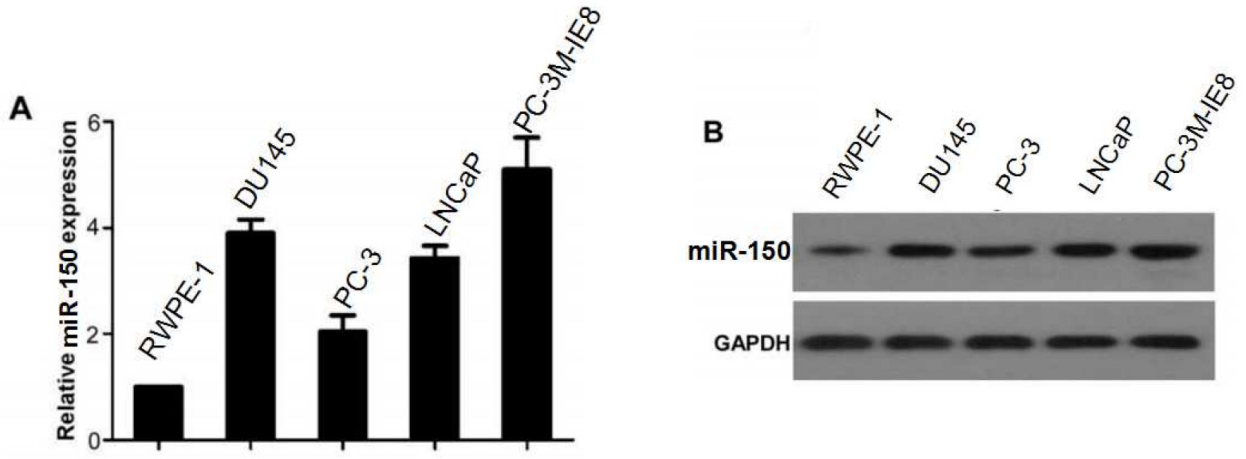

Figure 1. miR-150 mRNA and protein is overexpressed in PC cell lines. Expression of miR-150 mRNA and protein in PC cell lines (DU145, PC-3, LNCaP and PC-3M-IE8) and RWPE-1 were examined by quantitative RT-PCR **(A)** and Western blotting **(B).** The expression levels were normalized to GAPDH. Data is presented as mean±SD; P values were calculated using the Student's t-test and all were significant at $p<0.05$.

staining was graded as 0 (no staining), 1 (weak staining, light yellow), 2 (moderate staining, yellow brown), and 3 (strong staining, brown). We scored the proportion of positive tumor cells according to the following standards: 1 (<10%), 2 (10-35%), 3 (35-70%), and 4 (>70%). Then, the staining index was determined by multiplying the intensity score with the proportion score. Based on this method, we evaluated the miR-150 protein expression in PC tissue samples by staining index with scores of 0, 1, 2, 3, 4, 6, 8, 9, or 12. The cutoff score for high- and low-expression was determined based on a heterogeneity value measured through log-rank test with respect to OS. A staining index score of ≥6 was designated as tumors with high miR-150 expression,

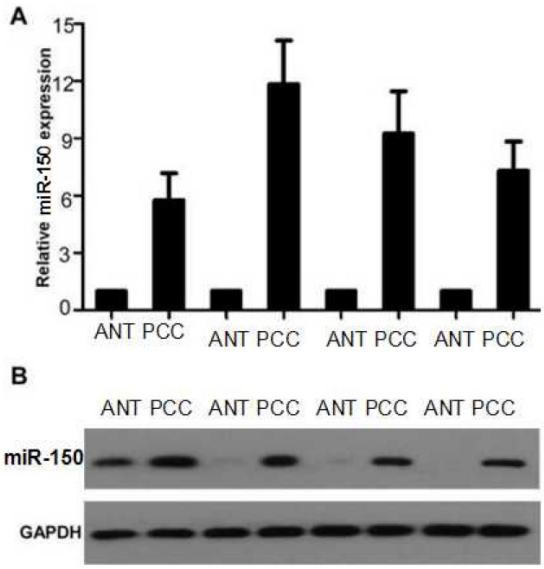

Figure 2. miR-150 mRNA and protein is overexpressed in PC tissue specimens. Expression of miR-150 mRNA and protein in 4 paired prostatic cancer and adjacent noncancerous tissues were tested by quantitative RT-PCR **(A)** and Western blotting **(B)**. The expression levels were normalized to GAPDH. Data is presented as mean±SD; P values were calculated using the Student's t-test and all were significant at p<0.01.

whereas a score of ≤4 was defined as low miR-150 expression.

Statistics

All of the statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The relationship between miR-150 protein expression and clinicopathological features was analyzed by the chi-square test. Kaplan-Meier method was used to estimate the OS and DFS, and the differences were compared using the log-rank test. HR values were calculated using the unadjusted univariate Cox regression analysis. Multivariate Cox regression analysis was used to test for independent prognostic indicators. All of the tests were two-sided, and statistical significance was set at $p < 0.05$.

Results

miR-150 is overexpressed in PC cell lines and tissue samples

To investigate the potential role of miR-150 in the tumorigenesis of PC, we firstly detected the mRNA and protein expression levels of miR-150 in 4 PC cell lines and one immortalized normal prostatic cell line RWPE-1 by qRT-PCR and Western blotting. The results showed that miR-150 expression was higher in PC cell lines compared with RWPE-1 cell line (p<0.05) (Figure 1A), and miR-150 protein was also overexpressed in PC cell lines compared with RWPE-1 cell line (p<0.05) (Figure 1B). To further explore the mRNA and protein expression of miR-150 in PC clinical specimens, we performed qRT-PCR and Western blotting in 4 pairs of PC and healthy tissue adjacent to tumor. The results demonstrated that miR-150 mRNA was expressed at markedly higher levels in all of the PC tissue samples compared with the adjacent nontumorous tissue samples (Figure 2A). Consistent with the qRT-PCR data, miR-150 protein was also overexpressed in PC tissues compared with the adjacent noncancerous tissues (p<0.01) (Figure 2B).

Correlation between miR-150 expression and clinicopathological features

To further evaluate the clinical significance of miR-150 in PC, the protein expression dynamics of miR-150 were detected using immunohistochemistry in 167 paraffin-embedded PC specimens. miR-150 protein was mainly expressed in the cytoplasm of PC cells (Figure 3). Overall, 108 of 167 (64.7%) samples were classified as tumors with high miR-150 expression, while 59 of 167 (35.5%) cases were designated as low miR-150 expression. This result clearly indicated that more than half of PC patients had significant overexpression of miR-150 in tumor tissues. The correlation between miR-150 expression and clinicopathological features of the PC patients are listed in Table 1. miR-150 expression was found to be significantly associated with tumor recurrence or metastasis (p=0.010). Patients with high miR-150 expression had a higher tendency to develop tumor recurrence or metastasis. No significant correlations were observed between miR-150 expression and any other clinicopathological features, such as gender, age, tumor size and number of tumor lesions.

High miR-150 expression is associated with poor survival

To evaluate the prognostic value of miR-150 expression in PC, survival curves were constructed using the Kaplan-Meier method, and differences were compared using the log-rank test. The results showed that PC patients with high miR-150 expression had significantly poorer OS (HR, 1.87; 95% CI, 1.19-2.94; p=0.006) and poorer DFS (HR,

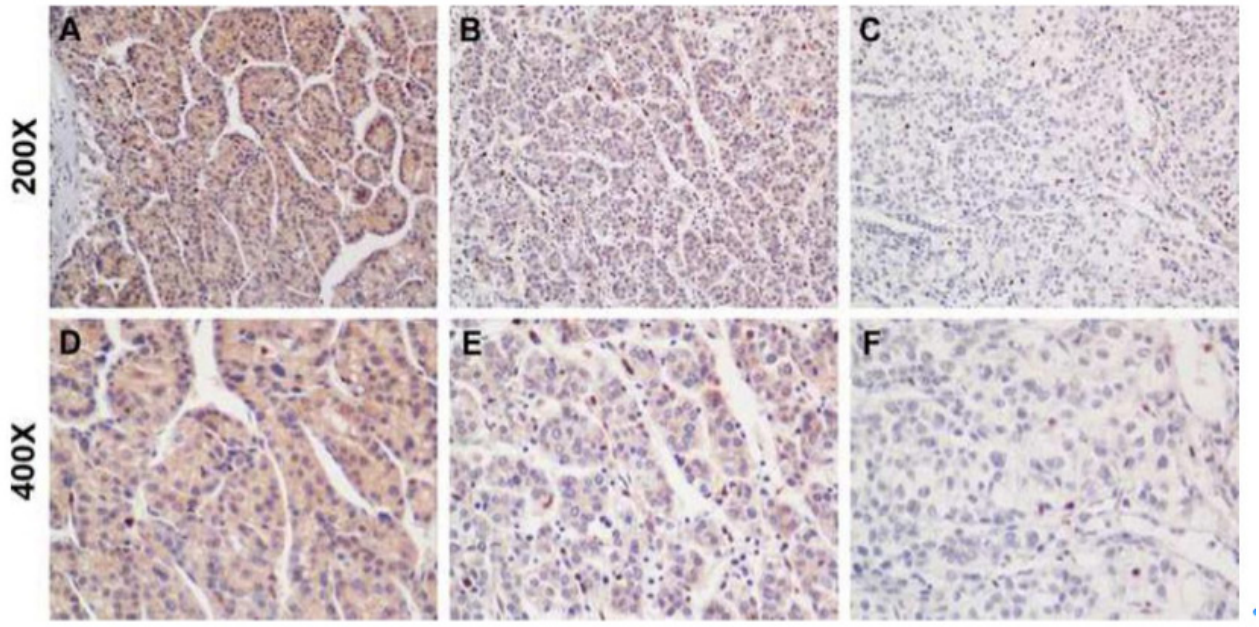

Figure 3. Immunohistochemical staining of miR-150 in PC tissues. miR-150 protein is mainly expressed in the cytoplasm of PC cells. Representative images of PC tissues with strong miR-150 staining **(A and C)**;moderate miR-150 staining **(C and D)**; and weak or negative detectable miR-150 staining **(E and F)**. Magnification is 200×for A, B and C and 400× for D, E and F.

Table 2. Univariate and multivariable Cox regression analysis of miR-150 expression level and overall survival and distant metastasis-free survival in patients with PC

Variables	*Univariate analysis*			*Multivariate analysis*		
	HR	*95% CI*	*p-value*	*HR*	*95% CI*	*p value*
			Overall survival			
miR-150 expression (low vs high)	1.87	1.19-2.94	0.006	1.81	1.14-2.87	0.012
Gender (male vs female)	1.49	0.83-2.67	0.184			
Age (≤50 vs >50 years)	1.14	0.75-1.71	0.545			
Tumor size (≤5 vs >5 cm)	1.71	1.04-2.83	0.036	1.95	1.17-3.23	0.01
Tumor lesions (multiple vs solitary)	1.61	1.07-2.42	0.024	1.7	1.3-4.14	0.012
Cirrhosis (present vs absent)	0.89	0.56-1.4	0.607			
			Distant metastasis-free survival			
miR-150 expression (low vs high)	1.9	1.21-2.98	0.005	1.84	1.16-2.93	0.01
Gender (male vs female)	1.53	0.85-2.74	0.157			
Age (≤50 vs >50 years)	1.12	0.74-1.68	0.597			
Tumor size (≤5 vs >5 cm)	1.71	1.03-2.83	0.037	1.99	1.2-3.31	0.008
Tumor lesions (multiple vs solitary)	1.7	1.13-2.55	0.012	1.81	1.2-2.73	0.005
Cirrhosis (present vs absent)	0.9	0.57-1.42	0.644			

HR: hazard ratio, CI: confidence interval

1.90; 95% CI, 1.21-2.98; p=0.005) than those with low miR-150 expression (Figure 4). The median OS and DFS in patients with high expression levels of miR-150 were 16.7 and 9.3 months, respectively, whereas they were 30.6 and 24.4 months, respectively, in patients with low expression levels of miR-150 (p<0.05). Furthermore, the cumulative 5-year OS was only 35.19% (95% CI, 26.18-44.20) in the high miR-150 expression group, whereas it was 55.93% (95% CI, 43.26-68.60) in the low miR-

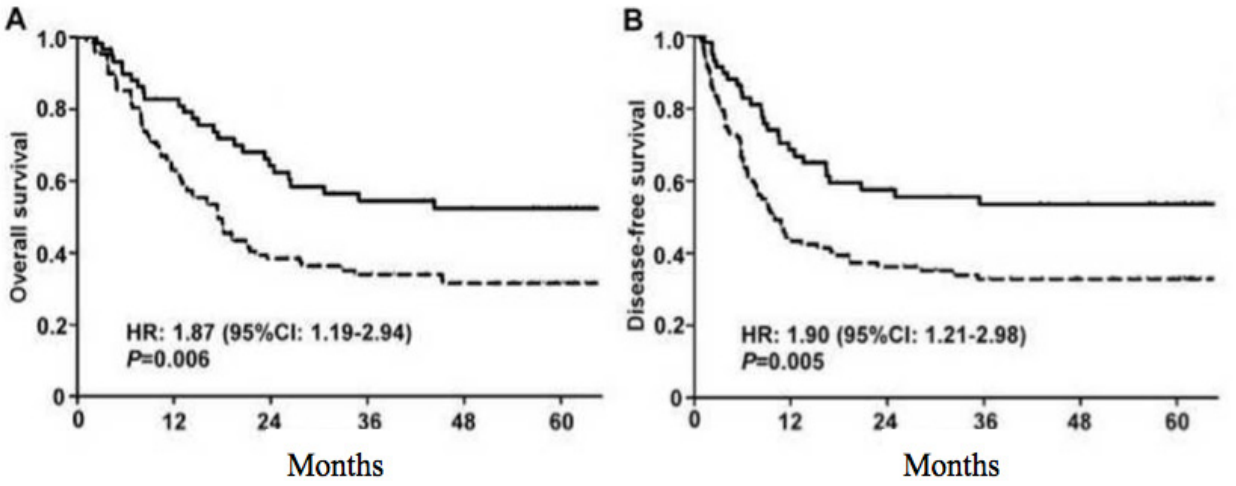

Figure 4. Kaplan-Meier survival curves for miR-150 patients stratified by miR-150 expression. **(A)** Overall survival. **(B)** Disease-free survival. CI:confidence interval; HR:hazard ratio; HR values were calculated using the unadjusted Cox proportional hazards model; P values were calculated using the unadjusted log-rank test.

150 expression group (p<0.05).

High miR-150 expression is an independent prognostic predictor of OS

Univariate and multivariate Cox regression analyses were performed to evaluate the impact of miR-150 expression and other clinicopathological features on survival in patients with PC. Univariate Cox regression analysis showed that miR-150 expression (p=0.006 and p=0.005, respectively), tumor size (p=0.036 and p=0.037, respectively), and number of tumor lesions (p=0.024 and p=0.012, respectively) were significantly associated with OS and DFS in patients with PC (Table 2). Furthermore, multivariate Cox regression analysis demonstrated that the expression of miR-150 (HR, 1.81; 95% CI, 1.14-2.87; p=0.012; HR, 1.84; 95% CI, 1.16-2.93; p=0.010, respectively), tumor size (HR, 1.95; 95% CI, 1.17-3.23; p=0.010; HR, 1.99; 95% CI, 1.20-3.31; p=0.008, respectively), and number of tumor lesions (HR, 1.70; 95% CI, 1.30-4.14; p=0.012; HR, 1.81; 95% CI, 1.20-2.73; p=0.005, respectively) were independent predictors of OS and DFS in patients with PC (Table 2).

Discussion

In this study, we examined miR-150 expression and its clinical significance in patients with PC for the first time. Our results showed that miR-150 was overexpressed in PC cell lines and healthy tissues at both the mRNA and protein levels. High miR-150 expression was positively correlated with tumor recurrence or metastasis. In addition, patients with high miR-150 expression had poorer OS and DFS. Taken together, these findings suggest that high miR-150 expression may serve as a novel prognostic biomarker to guide personalized therapy for PC patients.

Small RNAs including miRs have attracted attention as potential new tools for cancer therapeutic strategies [11,12]. Lots of relevant articles have reported the potential of targeting specific miRs in cancer therapy [13,14]. Recent studies demonstrate that miR-150 can function as an oncoprotein to promote malignant transformation and tumor growth [15]. Overexpression of miR-150 can promote the growth and invasiveness of tumor cell lines and enhance the mouse xenograft tumor growth *in vivo* [15]. Other studies, however, revealed that depletion of miR-150 expression accelerates the transformation and proliferation of tumor cell lines [5,16]. A recent research also reports that miR-150 plays important roles in regulating various biological processes during tumorigenesis [17]. However, in the present study, we firstly determined the expression levels of miR-150 mRNA and protein in several PC cell lines and healthy tissue samples using qRT-PCR and Western blotting. We found that miR-150 is obviously overexpressed in PC cell lines and tissue samples at both the transcriptional and translational levels, which is inconsistent with previous studies [5,16].

Precise prognosis assessment is critical for making appropriate therapeutic decisions, and clinical stage is the most important determinant

influencing the prognosis of PC patients. There are several staging systems available for PC patients, and the TNM staging system is the most prevalent one, which classifies PC patients into different risk groups based mainly on the clinicopathological characteristics. However, in clinical practice, the treatment outcomes of PC patients with the same clinical stage and receiving similar treatments display large variations, suggesting that the current staging systems could not correctly predict the prognosis of PC patients. Although multiple refinements of the TNM staging system have been performed, it is still difficult for surgeons to determine which individuals will experience relapse after curative surgical treatment. To overcome the limitations of the staging systems, various molecular biomarkers have been investigated and found to provide additional prognostic values. However, an ideal biomarker is still lacking. Recently, several studies demonstrated that miR-150 can function as prognostic biomarker in a range of tumors [5,16]. In this study, we defined miR-150 protein expression in a cohort of paraffin-embedded PC tissues using immunohistochemistry and analyzed its correlations with clinicopathological features and patients' survival. We found that PC patients with high miR-150 expression displayed a higher tendency to develop tumor recurrence or metastasis. In addition, PC patients with high miR-150 expression had poorer OS and DFS. More importantly, multivariate Cox regression analysis demonstrated that high miR-150 expression was an independent poor prognostic indicator for OS. Taken together, these results suggest that miR-150 could serve as a useful tool to identify PC patients at high risk of recurrence or metastasis who could benefit from more aggressive treatments.

In conclusion, for the first time, this study revealed that miR-150 was overexpressed in PC at both the mRNA and protein levels. PC patients with high miR-150 expression had a higher tendency to develop tumor recurrence or metastasis. Furthermore, high miR-150 expression served as an independent predictor of poor prognosis in PC patients. These results suggest that miR-150 may have important roles in the development and progression of PC, and can serve as a novel prognostic biomarker for guiding personalized therapy for PC patients.

References

1. Ferlay J, Parkin DM, Steliarova-Foucher E. Estimates of cancer incidence and mortality in Europe in 2008. Eur J Cancer 2010;46:765–781.
2. Lee JT, Lehmann BD, Terrian DM et al. Targeting prostate cancer based on signal transduction and cell cycle pathways. Cell Cycle 2008;7:1745-1762.
3. Valencia-Sanchez MA, Liu J, Hannon GJ et al. Control of translation and mRNA degradation by miRNAs and siRNAs. Genes Dev 2006;20:515-524.
4. Katada T, Ishiguro H, Kuwabara Y et al. microRNA expression profile in undifferentiated gastric cancer. Int J Oncol 2009;34:537-542.
5. Wang M, Yang W, Li M et al. Low expression of miR-150 in pediatric intestinal Burkitt lymphoma. Exp Mol Pathol 2014;96:261-266.
6. Katada T, Ishiguro H, Kuwabara Y et al. microRNA expression profile in undifferentiated gastric cancer. Int J Oncol 2009;34:537-542.
7. Amaral FC, Torres N, Saggioro F et al. MicroRNAs differentially expressed in ACTH-secreting pituitary tumors. J Clin Endocrinol Metab 2009;94:320-323.
8. Ma Y, Zhang P, Wang F et al. miR-150 as a potential biomarker associated with prognosis and therapeutic outcome in colorectal cancer. Gut 2012;61:1447-1453.
9. Yokobori T, Suzuki S, Tanaka N et al. MiR-150 is associated with poor prognosis in esophageal squamous cell carcinoma via targeting the EMT inducer ZEB1. Cancer Sci 2013;104:48-54.
10. Chang TC, Yu D, Lee YS et al. Widespread microRNA repression by Myc contributes to tumorigenesis. Nat Genet 2008;40:43-50.
11. Pecot CV, Calin GA, Coleman RL et al. RNA interference in the clinic: challenges and future directions. Nat Rev Cancer 2011;11:59-67.
12. Soifer HS, Rossi JJ, Saetrom P. MicroRNAs in disease and potential therapeutic applications. Mol Ther 2007;15:2070-2079.
13. Elmén J, Lindow M, Schütz S et al. LNA-mediated microRNA silencing in non-human primates. Nature 2008;452:896-899.
14. Silvestri P, Di Russo C, Rigattieri S et al. MicroRNAs and ischemic heart disease: towards a better comprehension of pathogenesis, new diagnostic tools and new therapeutic targets. Recent Pat Cardiovasc Drug Discov 2009;4:109-118.
15. Zhang N, Wei X, Xu L. miR-150 promotes the proliferation of lung cancer cells by targeting P53. FEBS Lett 2013;587:2346-2351.
16. Yokobori T, Suzuki S, Tanaka N et al. MiR-150 is associated with poor prognosis in esophageal squamous cell carcinoma via targeting the EMT inducer ZEB1. Cancer Sci 2013;104:48-54.
17. Huang S, Chen Y, Wu W et al. miR-150 promotes human breast cancer growth and malignant behavior by targeting the pro-apoptotic purinergic P2X7 receptor. PLoS One 2013;8:e80707.