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Tumor and Stem Cell Biology

Cancer Research

Paxillin Predicts Survival and Relapse in Non–Small Cell Lung Cancer by MicroRNA-218 Targeting

De-Wei Wu[1], Ya-Wen Cheng[2], John Wang[3], Chih-Yi Chen[4], and Huei Lee[1,2,5,6]

Abstract

Paxillin (PXN) gene mutations are associated with lung adenocarcinoma progression and PXN is known to be a target gene of microRNA-218 (miR-218). On this basis, we hypothesized that PXN overexpression via miR-218 suppression may promote tumor progression and metastasis and that PXN may predict survival and relapse in non–small cell lung cancer (NSCLC). Expression of miR-218 and PXN in 124 surgically resected lung tumors were evaluated by real-time PCR and immunohistochemical analysis. The prognostic value of miR-218 and PXN expression on overall survival (OS) and relapse-free survival (RFS) was analyzed by the Kaplan–Meier test and Cox regression analysis. miR-218 expression in lung tumors was negatively associated with PXN expression. Multivariate analyses showed that PXN and miR-218 might independently predict OS and RFS, respectively, in NSCLC. Moreover, patients with low miR-218 combined with PXN-positive had the worst OS and RFS among the 4 combinations. In a cell model, PXN was negatively regulated by miR-218 and cell proliferation, invasion, and soft agar colony formation were enhanced by PXN overexpression induced by miR-218 suppression. Taken together, our findings suggest that PXN overexpression induced by miR-218 suppression is an independent predictor of survival and relapse in NSCLC, highlighting PXN as a potential therapeutic target to improve clinical outcomes in this disease. *Cancer Res; 70(24); 10392–401. ©2010 AACR.*

Introduction

Lung cancer is the leading cause of cancer deaths in industrial countries, with only 15% of all affected individuals surviving more than 5 years from the time of diagnosis (1). Tumor migration and metastasis are the key causes of death in patients with poor prognosis (2). However, the molecular pathogenesis of this disease remains largely unclear.

The focal adhesion protein paxillin (PXN), a target of several genes, is involved in signal transduction and is important for cell mobility and migration. An early study showed that PXN might suppress lung tumor progression (3). Recently, about 10% of PXN mutations were found in lung tumors from Caucasians and African-Americans but not from Taiwanese patients (4). The authors further showed that mutated PXN might act as an oncogene that enhances cell proliferation *in vitro* and causes xenograft tumors *in vivo*. Interestingly, PXN overexpression has been observed in lung cancer cells and tumor tissues (4). In human cervical cancer tissues, human papillomavirus (HPV) 18-immortalized genital epithelial cells, and high-grade dysplastic and invasive cervical carcinomas, PXN overexpression may be associated with cervical tumor metastasis (5). However, the underlying mechanism of PXN overexpression involved in human tumorigenesis is not yet known.

MicroRNAs (miRNAs) are small noncoding RNAs that serve as negative regulators of gene expression (6–8). By integration with the 3′-untranslated region (3′-UTR) of mRNA, via partial sequence homology, miRNAs cause gene silencing either by mRNA degradation or by repression of translation (9). Some reports have indicated that miRNAs are key players in the regulation of tumor proliferation and cell invasion (10–12). The suppression of miR-218 has been shown in lung tumors (13). Recent report indicated that miR-218 expression was decreased in primary normal bronchial epithelial (NHBE) cells after exposure to cigarette smoke condensates and 1 of the 85 genes known to be targeted by miR-218 is PXN (14). In addition, miR-218 also seems to function as a tumor suppressor to inhibit cancer cell proliferation and invasion (15, 16). These observations prompt us to assume that PXN expression is regulated by miR-218 expression and that PXN overexpression may participate in lung tumor progression and metastasis.

In this study, 124 lung tumors surgically resected from lung cancer patients were evaluated for PXN and miR-218 expression by immunohistochemical analysis and real-time

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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PCR, and the prognostic value of PXN and miR-218 expression was evaluated with respect to overall survival (OS) and relapse-free survival (RFS). Data were statistically analyzed by Kaplan–Meier and multivariate Cox regression analyses. We further examined the cell proliferation rate, efficacy of colony formation, invasion, and soft agar colony formation to verify whether PXN upregulation induced by miR-218 suppression could predict a poor clinical outcome resulting from enhanced tumor growth and metastasis.

Materials and Methods

Study subjects and cell lines

Lung tumor specimens were collected from 124 patients with primary lung cancer at the Department of Thoracic Surgery, Taichung Veterans General Hospital, between 1998 and 2004. The patients were requested to submit a written informed consent form approved by the institutional review board. The tumor type and stage of each collected specimen were histologically determined according to the World Health Organization's (WHO) classification system. Forty female (32.2%), 84 males (67.8%), 66 nonsmokers (53.2%), 58 smokers (46.8%), 53 patients with adenocarcinoma (42.7%), 71 patients with squamous cell carcinoma (57.3%), 50 patients with stage I (40.3%), 25 patients with stage II (20.2%), and 49 patients with stage III (39.5%) were enrolled in this study (Supplementary Table 1).

Cell lines

A549, H1299, Ch27, H460, Calu-1, H661, SiHa, and C33A cells were obtained from the American Type Culture Collection (ATCC) and cultured as described. CL1-0 and CL1-5 cells were kindly provided by Dr. P.-C. Yang (Department of Internal Medicine, National Taiwan University Hospital). TL-1, TL-2, and TL-4 were kindly provided by Dr. Y.-W. Cheng (Institute of Medicine, Chung Shan Medical University; refs. 17, 18). Cells were cultured and stored according to the suppliers' instructions and used at passages 5 to 20. Once resuscitated, cell lines were routinely authenticated (once every 6 months, cells were last tested in December 2009) through cell morphology monitoring, growth curve analysis, species verification by isoenzymology and karyotyping, identity verification using short tandem repeat profiling analysis, and contamination checks.

Real-time quantitative RT-PCR analysis of miR-218 and PXN mRNA expression levels

DNase I–treated total RNA (10 ng) was subjected to miRNA RT-PCR analysis with the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems), miRNA Assays (Applied Biosystems), and a Real-Time Thermocycler 7500 (Applied Biosystems). RNU6B was used as the small RNA reference housekeeping gene. For PXN and SLIT2 mRNA expression, the primers used for RT-PCR analysis are described in Supplementary Table 2. Reverse transcription reaction and real-time quantitative PCR were done as described previously (17). The miR-218 and PXN mRNA levels in lung tumors that were higher than the median value were defined as "high," whereas levels lower than the median value were defined as "low."

Immunohistochemical analysis

Antimouse PXN antibodies were purchased from Neomarkers. The immunohistochemical procedures and quantification methods were as described previously (18). In lung tumors, immunostaining was defined as "positive" if PXN immunoreactivity was observed in 10% or more of the cells in paraffin sections; tumors with lower percentages of immunoreactive cells showed "negative" immunostaining.

miR-218 precursor and inhibitor transfection

Cells were grown to confluence in 6-well plates. miR-218 precursor (Pre-miR-218, 20–40 nmol/L/well; (Ambion), miR-218 inhibitor (40–80 nmol/L/well; (Ambion), and negative control (Ambion) cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. Transfection efficiency was evaluated by real-time PCR.

Luciferase reporter assay

Double-stranded oligonucleotides corresponding to the wild-type (WT 3′-UTR) or mutant (Mut 3′-UTR) miR-218 binding site in the 3′-UTR of PXN were synthesized and ligated between the *Spe*I and *Hin*dIII restriction sites of pmiR-REPORT miRNA Expression Reporter Vector (Ambion). The oligonucleotides used are described in Supplementary Table 2. Cells were transfected with appropriate plasmid and Pre-miR-218. Luciferase assays were done using the luciferase reporter assay system (Promega) 48 hours after transfection. Normalized luciferase activity was reported as luciferase activity/β-galactosidase activity.

Plasmid construction

Detailed plasmids are presented in the Supplementary Methods section.

HPV16 E6 siRNA transfection assays

The RNA interference target sequences for HPV16 E6 siRNA (E6si) have been previously verified (19, 20). The procedures and methods were as described previously (17, 18).

Western blotting assay

For immunoblotting of PXN, β-actin, and HPV16 E6 cell lysates were prepared as described above (18).

Doubling time and soft agar assays

The procedures and methods of doubling time and soft agar assays were as described previously (17, 18).

Colony formation assay

For the colony formation assay, 200 transfected cells were plated in a 6-well plate for 10 days. Colonies were fixed with methanol/acetone (1:1) and stained with crystal violet (1 mg/mL).

Invasion assay

These assays were done according to a previously reported method (21).

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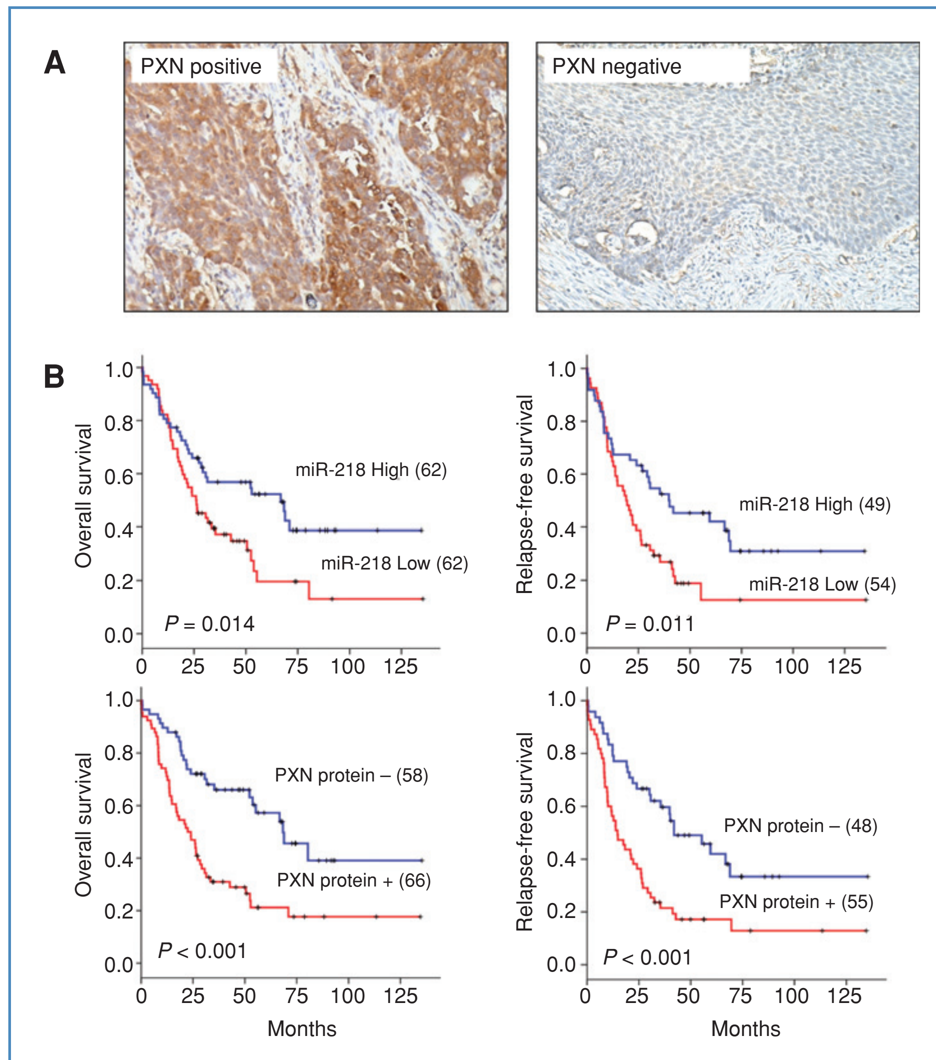

Figure 1. When miR-218 expression was low and PXN expression was high, the clinical outcome in lung cancer patients was poor. A, representative immunostained images showing high PXN expression (left) and low PXN expression. B, OS and RFS curves for all studied patients with high or low miR-218 expression (top) and high or low PXN expression (bottom).

Statistical analysis

Statistical analysis was conducted using the SPSS statistical software program (Version 15.0; SPSS Inc.). The association between miR-218 expression and PXN protein expression was analyzed by the χ^2 test. Survival plots were generated using the Kaplan–Meier method, and differences between patient groups were determined by the log-rank test. Multivariate Cox regression analysis was conducted to determine OS and RFS. The analysis was stratified for all known variables (age, gender, smoking status, tumor type, and tumor stage) and protein expression.

Results

PXN expression in lung tumors is correlated with miR-218

To understand whether PXN expression in lung tumors is associated with miR-218 expression, the expression of miR-218, PXN mRNA, and protein were evaluated by real-time PCR and immunohistochemical analysis. Representative PXN protein expression in the lung tumors is shown in Figure 1A, which shows that PXN was predominantly expressed in the cytoplasm of the tumor cells and that some PXN protein was found in the nucleus of the tumor cells. In this studied population, the prevalence of PXN mRNA and protein expression in lung tumors was positively correlated with tumor stage; namely, the high or positive expression of PXN mRNA and protein was more prevalent in stage III patients than in stage I and II patients (PXN mRNA, $P = 0.022$; PXN protein, $P = 0.035$; Table 1). In contrast, the high expression of miR-218 in stage III patients was marginally lower than that in stage I and II patients ($P = 0.055$; Table 1). The expression of PXN mRNA and protein in lung tumors was negatively correlated with miR-218 expression (PXN mRNA, $P = 0.012$; PXN protein, $P = 0.031$; Table 1). These results suggest that PXN expression in lung tumors is negatively associated with miR-218 expression.

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Table 1. The correlation between clinical parameters with miR-218 and PXN expressions and correlation between miR-218 and the expression of PXN mRNA and protein

		miR-218			PXN mRNA			PXN protein		
	No	Low	High	*P*	Low	High	P	Negative	Positive	*P*
Age										
<68	59	29	30	0.857	31	28	0.590	28	31	0.884
≧68	65	33	32		31	34		30	35	
Gender										
Female	40	22	18	0.442	18	22	0.442	14	26	0.070
Male	84	40	44		44	40		44	40	
Smoking status										
Nonsmokers	66	32	34	0.719	30	36	0.280	26	40	0.079
Smokers	58	30	28		32	26		32	26	
Tumor type										
ADC	53	31	22	0.102	22	31	0.102	18	35	0.013
SCC	71	31	40		40	31		40	31	
Stage										
I	50	24	26	0.055	30	20	0.022	27	23	0.035
II	25	8	17		15	10		15	10	
III	49	30	19		17	32		16	33	
miR-218										
Low	62				24	38	0.012	23	39	0.031
High	62				38	24		35	27	
PXN mRNA										
Low	62							39	23	<0.001
High	62							19	43	

ADC, adenocarcinoma; SCC, squamous cell carcinoma.

PXN and miR-218 are associated with OS and RFS in lung cancer

We hypothesized that PXN overexpression in lung tumors due to miR-218 suppression could contribute to tumor progression and metastasis. Therefore, we expected that PXN and miR-218 expression would be associated with OS and RFS in lung cancer. Of the 124 patients enrolled, 103 were available for RFS analysis, with a median number of follow-up months of 26.0. In the RFS analysis group, the condition relapsed in 41 patients (5, local recurrence; 27, distant metastasis; 9, local recurrence and distant metastasis) and 32 patients died from the disease. No patients received adjuvant treatment before surgical therapy. Kaplan–Meier analysis showed that patients with low miR-218 expression had shorter median months of OS and RFS than did patients with high miR-218 expression (26.0 months vs. 33.9 months, $P = 0.014$ for OS; 19.3 months vs. 35.4 months, $P = 0.011$ for RFS; Fig. 1B). In addition, we observed that PXN-positive patients had shorter median months of OS and RFS than did PXN-negative patients (23.2 months vs. 38.4 months, $P < 0.001$ for OS; 14.7 months vs. 36.4 months, $P < 0.001$ for RFS; Fig. 1B). As expected, the median months of OS and RFS were significantly higher for stage I patients than for stage II or III patients (35.8 months for stage I vs. 30.6 months for stage II vs. 17.1 months for stage III, $P < 0.001$ for OS; 33.8 months for stage I vs. 26.5 months for stage II vs. 12.1 months for stage III, $P < 0.001$ for RFS; Supplementary Table 3). Interestingly, in this studied population, patients with adenocarcinomas had shorter median months of OS and RFS than did those with squamous cell carcinomas (22.0 months vs. 40.4 months, $P = 0.002$ for OS; 18.9 months vs. 30.6 months, $P = 0.007$ for RFS; Supplementary Table 3). Multivariate analysis conducted after the parameters of age, gender, smoking, tumor type, and tumor stage were adjusted, showed that the hazard ratios (HR) for OS and RFS in patients with low miR-218 were 1.67 (OS) and 2.08 (RFS) times those of patients with high miR-218, respectively (95% CI = 1.03–2.70, $P = 0.036$ for OS; 95% CI = 1.23–3.50, $P = 0.006$ for RFS; Table 2).

As expected, patients with PXN-positive expression had HRs that were 2.62 (OS) and 2.28 (RFS) times those of patients with PXN-negative expression (95% CI = 1.58–4.37, $P < 0.001$ for OS; 95% CI = 1.37–3.77, $P = 0.001$ for RFS; Table 2). Moreover, patients with low miR-218 and PXN-positive expression had the worst OS and RFS among the 4 possible combinations (HR = 3.94, 95% CI = 1.93–8.02, $P < 0.001$ for OS; HR = 3.84, 95% CI = 1.93–7.67 for RFS, $P < 0.001$; Table 2). These results suggest that suppression of miR-218 may induce PXN overexpression to promote tumor malignancy in patients and lead to poor OS and RFS.

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Table 2. Cox regression analysis of various potential prognostic factors in lung cancer patients with miR-218 and PXN expression

Variables	Case no.	Median survival, mo	OS, %	Adjusted HR[a]	95%CI	*P*	Case no.	Median survival, mo	RFS, %	Adjusted HR[a]	95%CI	*P*
miR-218												
+ (High)	62	33.9	48.4	1			49	35.4	38.8	1		
− (Low)	62	26.0	29.0	1.67	1.03–2.69	0.036	54	19.3	20.4	2.08	1.23–3.50	0.006
PXN												
− (Negative)	58	38.4	55.2	1			48	36.4	43.8	1		
+ (Positive)	66	23.2	24.2	2.62	1.58–4.37	<0.001	55	14.7	16.4	2.28	1.37–3.77	0.001
miR-218/PXN												
+/−	35	52.0	65.7	1			27	40.0	48.1	1		
−/−	23	32.4	39.1	1.93	0.88–4.25	0.102	21	30.6	38.1	1.70	0.77–3.73	0.188
+/+	27	27.3	25.9	3.13	1.48–6.64	0.003	22	25.5	27.3	1.88	0.87–4.05	0.107
−/+	39	18.3	23.1	3.94	1.93–8.02	<0.001	33	13.8	9.1	3.84	1.93–7.67	<0.001

[a]HR adjusted for age, gender, smoke, tumor type, and tumor stage.

PXN transcription is modulated by miR-218 in lung cancer cells

To explore whether PXN expression is negatively associated with miR-218 expression in lung cancer cells, 11 lung cancer cell lines and 2 cervical cancer cells lines were used to evaluate PXN and miR-218 expression by real-time PCR. The expression levels of PXN mRNA in all of these cell lines were inversely correlated with the miR-218 expression levels (Fig. 2A). To verify whether PXN mRNA expression was modulated by miR-218, CL1-5 and TL-1 cells, which have high levels of PXN mRNA, were treated with a miR-218 precursor. In addition, CL1-0 and TL-4 cells, which have low levels of PXN mRNA, were treated with an miR-218 inhibitor. After confirming the expression of mature miR-218 in treatment cells evaluated by real-time PCR (Fig. 2B), we found that the PXN mRNA levels in the CL1-5 and TL-1 cells were reduced by the miR-218 precursor in a dose-dependent manner whereas the levels in the CL1-0 and TL-4 cells were markedly increased in response to the miR-218 inhibitor (Fig. 2C). To obtain further direct evidence that PXN is a target of miR-218, the miR-218 binding sequences of the WT or Mut PXN 3'-UTR (Fig. 2D) were constructed with a pmiR-REPORT miRNA Expression Reporter Vector (Ambion) and subsequently transfected into TL-1 and CL1-5 cells, respectively. The luciferase reporter assay showed that the reporter activity of WT-PXN 3'-UTR was markedly reduced by miR-218 in both cell types, but no reduction was seen in the reporter activity of Mut-PXN 3'-UTR when compared with miR-non-specific control (NC) cells (Fig. 2D). These results suggest that miR-218 may modulate PXN expression by directly targeting its 3'-UTR in lung cancer cells.

Overexpression of PXN following miR-218 suppression enhances cell proliferation, soft agar colony formation, and invasion ability

Alterations in PXN expression have been shown to be implicated in cell growth and invasion in lung cancer (4). To determine whether PXN upregulation by a reduced expression of miR-218 can increase cell proliferation, soft agar colony formation, and invasion ability, CL1-5 cells were treated with PXN RNAi and an miR-218 precursor, and CL1-0 cells were treated with a miR-218 inhibitor. The doubling time of CL1-5 cells was significantly elevated by treatment with the miR-218 precursor (from 23.8 hours in the control cells to 35.2 hours) and PXN RNAi [from 23.8 hours in the nonspecific RNAi control cells (NC) to 34.7 hours; Fig. 3A]. Similarly, the doubling time of CL1-0 cells was reduced by treatment with the miR-218 inhibitor in a dose-dependent manner (from 23.2 hours in the control cells to 22.1 and 19.2 hours; Fig. 3A). Additionally, the colony count was found to depend on PXN expression in miR-218 expression-altered or PXN-knockdown cells (Fig. 3B). Soft agar assay indicated that colony size was markedly decreased in CL1-5 cells after treatment with the miR-218 precursor and PXN RNAi, and that colony formation efficacy was also reduced (Fig. 3C). A Boyden chamber assay indicated that the invasion ability of CL1-5 cells was significantly reduced by treatment with the miR-218 precursor and PXN RNAi (Fig. 3C). On the other hand, the invasion ability and colony formation efficacy of CL1-0 cells was markedly increased by treatment with the miR-218 inhibitor (Fig. 3C).

To verify whether PXN is responsible for cell growth and metastasis, CL1-0 cells were treated with PXNsi or PXNsi and the miR-218 inhibitor. The results of the Boyden chamber and colony formation assays showed that the oncogenic potential decreased markedly after treatment with both agents when compared with the potential after treatment with the miR-218 inhibitor alone. Western blotting indicated that the oncogenic potential of CL1-0 cells treated with different agents was consistent with PXN expression (Fig. 3D). These results clearly indicate that upregulation of PXN by miR-218 suppression promotes the cell proliferation and oncogenic potential in lung cancer cells.

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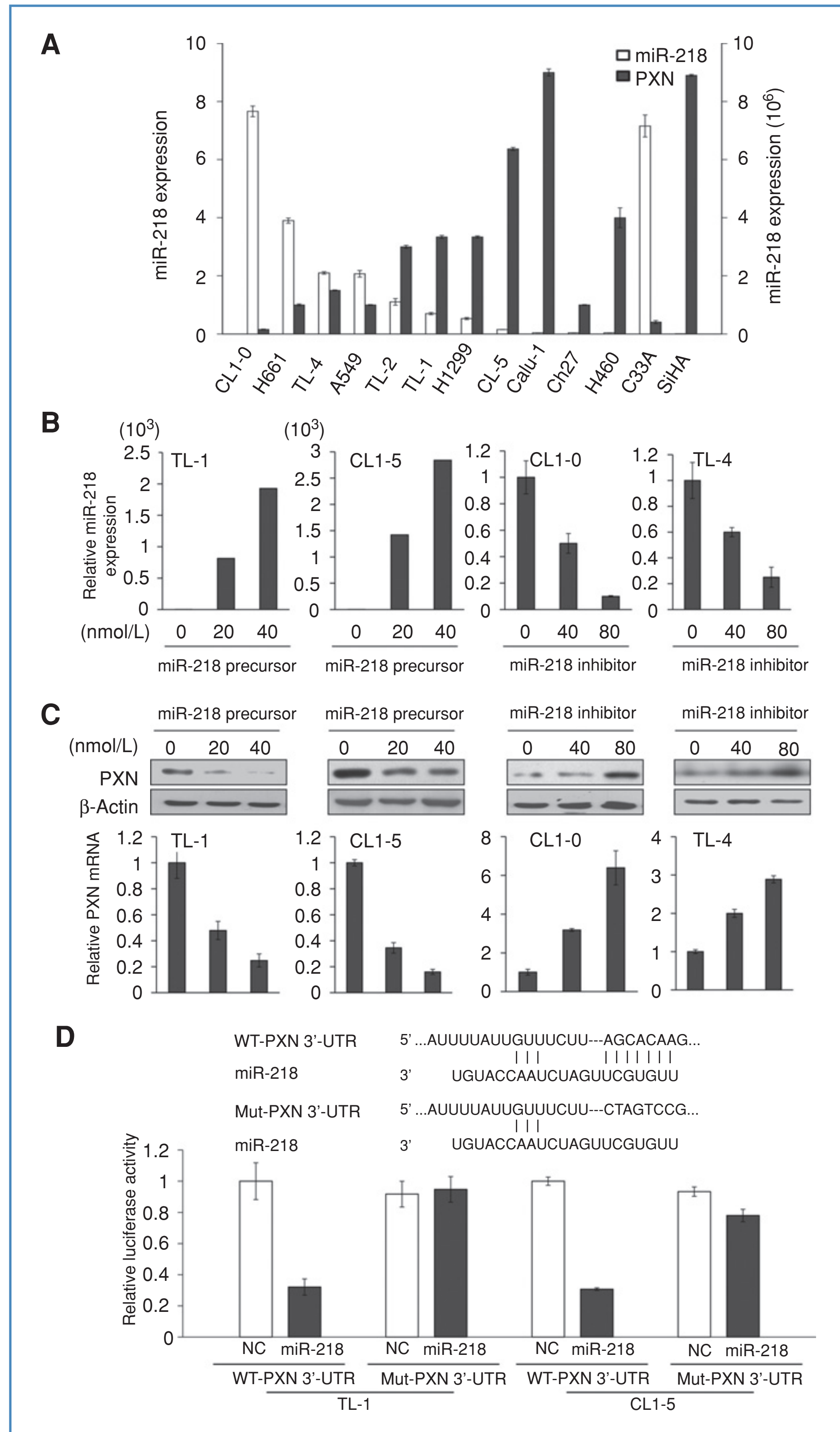

Figure 2. miR-218 suppressed PXN expression by directly targeting its 3'-UTR. A, expression levels of miR-218 and PXN were evaluated by real-time PCR in 11 lung and 2 cervical cancer cell lines. B, in TL-4 and CL1-0 cells, miR-218 was knocked down by a miR-218 inhibitor (at 2 doses). miR-218 was overexpressed by treatment with a miR-218 precursor (various doses) in TL-1 and CL1-5 cells. The miR-218 level was determined by real-time PCR. C, PXN mRNA level was determined by real-time PCR, and the levels of PXN and β-actin protein were evaluated by Western blotting. β-Actin was used as a protein loading control. NC, nonspecific control. D, top, miR-218 binding sequence of WT or Mut PXN 3'-UTR were synthesized and ligated with pmiR-REPORT miRNA Expression Reporter Vector. Bottom, TL-1 and CL1-5 cells were transfected with Pre-miR-218 (40 μmol/L/well; Ambion), miR-nonspecific control (Ambion), 500 ng pMIR-Reporter luciferase vector, including 3'-UTR of PXN (with WT or Mut miR-218 response element), and β-galactosidase plasmid. In all experiments, the relative level in the NC and vector controls was arbitrarily assigned as 1.

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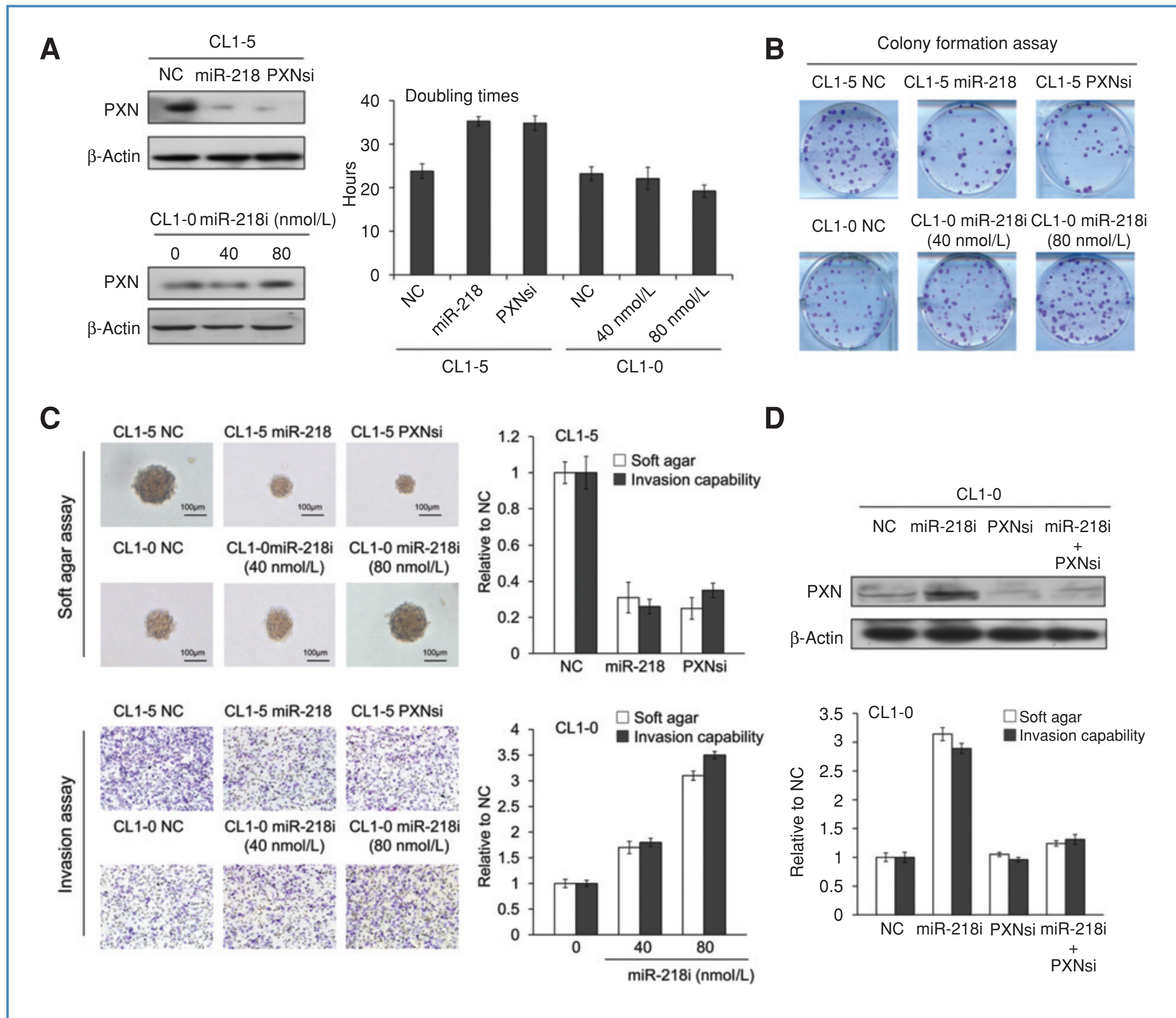

Figure 3. PXN is deregulated by miR-218 and cell proliferation and oncogenic potential are enhanced. A, proliferation rate of CL1-0 cell with or without miR-218 inhibitor treatment and of CL1-5 cells with or without PXN-knockdown plasmid and miR-218 precursor treatment were evaluated on the basis of the cell doubling time. The change in the PXN expression was confirmed by Western blotting (left), and the doubling time is shown at the right. B, representative colony counts of CL1-0 cells with or without miR-218 inhibitor treatment and CL1-5 cells with or without PXN-knockdown plasmid and miR-218 precursor treatment. C, representative soft agar colony sizes and number of invading cells for CL1-0 cells with or without miR-218 inhibitor treatment and CL1-5 cells with or without PXN-knockdown plasmid and miR-218 precursor treatment (left). The soft agar colony formation and invasion ability were evaluated for CL1-0 cells with or without miR-218 inhibitor treatment and for CL1-5 cells with or without PXN-knockdown plasmid and miR-218 precursor treatment and compared with those of NC controls (right). D, effects of PXNsi, miR-218 inhibitor, and combined treatment with PXNsi and the miR-218 inhibitor on PXN expression, soft agar colony formation, and invasion ability in CL1-0 cells. NC, nonspecific control; miR-218i, miR-218 inhibitor. In all experiments, the relative mRNA level in the NC controls was arbitrarily assigned as 1.

PXN modulation by E6 is mediated by miR-218

Expression of miR-218 has been shown to be reduced by HPV16 E6 in cervical cancer (22). Therefore, we then questioned whether E6 in HPV16-positive lung cancer cells could have a similarly inhibitory effect on miR-218 expression. To this end, E6 in TL-1 cells was knocked down using E6 RNAi, and E6 was overexpressed in TL-4 cells by transfection with an E6 cDNA plasmid. As expected, miR-218 expression in the TL-1 cells was elevated by E6 knockdown in a dose-dependent manner (Fig. 4A). Conversely, miR-218 expression in the TL-4 cells was significantly decreased due to E6 overexpression (Fig. 4A). The increase in the miR-218 level due to E6 knockdown in the TL-1 cells concomitantly decreased PXN expression (Fig. 4A). The opposite relationship was observed with E6 overexpression in the TL-4 cells (Fig. 4A). To verify whether PXN expression is modulated by E6 via miR-218, TL-1 cells were treated with E6si and the miR-218 inhibitor either separately or concomitantly. Real-time PCR and Western blot data showed that the PXN mRNA level was greatly reduced by E6 knockdown and slightly elevated by treatment with the

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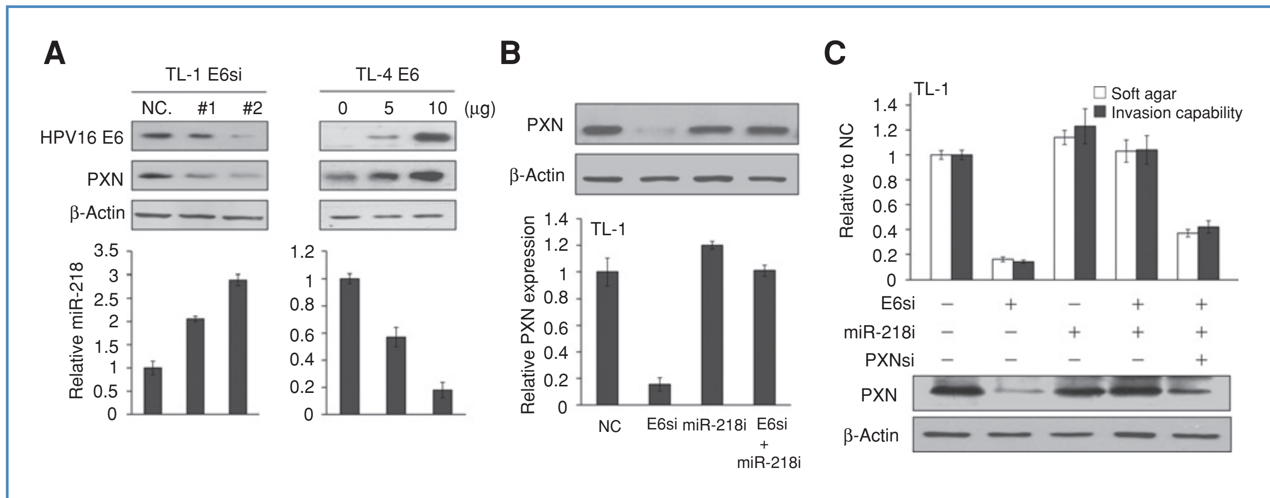

Figure 4. PXN is an indicator of HPV-induced increased oncogenic potential. A, HPV16 E6 in TL-1 cells was knocked down by 2 E6 siRNAs (E6si#1 and #2). HPV16 E6 was overexpressed using an E6 overexpression plasmid (at various doses) in TL-4 cells. The miR-218 level was determined by real-time PCR, and the levels of HPV16 E6, p53, PXN, and β-actin were evaluated by Western blotting. β-Actin was used as a protein loading control. NC, nonspecific control. B, TL-1 cells were transfected with E6 siRNA and the miR-218 inhibitor, as indicated. The total amount of siRNA and miRNA inhibitor was kept constant by addition of the negative control in each transfection unit. C, effects of PXNsi, the miR-218 inhibitor, and E6si on PXN expression, soft agar colony formation, and invasion ability in TL-1 cells. NC, nonspecific control; miR-218i, miR-218 inhibitor. In all experiments, the relative mRNA level in NC controls was arbitrarily assigned as 1.

miR-218 inhibitor as compared with levels in the nonspecific RNAi control (NC) cells (Fig. 4B). However, the PXN mRNA level in the TL-1 cells remained unchanged with the combined treatment with E6si and the miR-218 inhibitor (Fig. 4B). These results clearly indicate that PXN expression modulation by E6 is predominantly mediated by miR-218. Further verification of the role of PXN on the oncogenic potential of cells was obtained by treating TL-1 cells with E6si, the miR-218 inhibitor, PXNsi, and combinations of these 3 agents. The oncogenic potential was greatly reduced by E6si, followed by the combination treatments; conversely, the oncogenic potential was slightly elevated by treatment with the miR-218 inhibitor and the combined E6si and miR-218 inhibitor treatment (Fig. 4C). These results clearly indicate that PXN upregulated by E6 is mediated by miR-218 in lung cancer cells.

Discussion

In recent years, miRNA molecules have emerged as powerful regulators of gene expression and genome stability. Several miRNAs have been found to be involved in the initiation and progression of lung cancer and to be associated with the diagnosis, prognosis, and treatment of lung cancer. For diagnosis, miR-205 is a highly accurate marker for lung cancer of squamous histology (23), whereas miR-488, miR-503, and miR-647 have been identified as markers of disease recurrence in stage I lung cancer (24). With regard to prognosis, miR-155, let-7a, miR-221, miR-137, miR-372, miR-182, miR-21, miR-205, and miR-34a have been found useful in predicting survival and relapse in lung cancer patients (13, 25–28). As shown in this study, miR-218 may predict the OS and RFS in lung cancer patients, although the prognostic significance of miR-218 for OS and RFS was relatively lower than that of PXN (HR: 1.67 vs. 2.62 for OS; 2.08 vs. 2.28 for RFS; Table 2). HPV16 E6 has been shown to reduce miR-218 expression in cervical cancer cells (22). HPV16/18 infection was found to be associated with lung cancer development in a group of Taiwanese women, more than 90% of whom had never smoked (29). We also found that E6 was negatively associated with miR-218 expression and positively associated with PXN expression ($P = 0.001$ for miR-218; $P < 0.001$ for PXN; Supplementary Table 4). The HRs for RFS with regard to miR-218 and PXN expression were also elevated in E6-positive patients compared with E6-negative patients (3.38 vs. 2.09 for miR-218; 2.79 vs. 2.43 for PXN; Supplementary Table 5). Additionally, the HRs for OS and RFS in patients with $E6^{+}$/miR-218^{-}/PXN^{+} were markedly increased to 6.68 and 5.97 times those in patients with $E6^{-}$/miR-218^{+}/PXN^{-}, respectively (95% CI = 2.44–18.30, $P < 0.001$ for OS; 95% CI = 2.01–17.75, $P = 0.001$ for RFS; Supplementary Table 5). The results suggest that PXN overexpression induced by miR-218 suppression may promote tumor malignancy, resulting in poor OS and RFS, and it may also be useful as an indicator of OS and RFS in cases of lung cancer with HPV infection.

PXN is a multidomain protein that localizes at the intracellular surfaces where cells adhere to the extracellular matrix. Through the interactions of its multiple protein-binding modules, many of which are regulated by phosphorylation (30–32), PXN serves as a platform for the recruitment of numerous regulatory and structural proteins that control the dynamic changes in cell adhesion, cytoskeletal reorganization (33–35), and gene expression that are necessary for cell survival and metastasis (5, 34). A recent report indicated that PXN mutation and gene amplification in lung cancer cells and tumor

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tissues might promote cell growth and invasion (4). In this study, PXN overexpression in lung cancer cells and tumor tissues was also found to enhance cell proliferation and tumor invasiveness (Fig. 3). Patients with high PXN expression had poorer OS and RFS than those with low PXN expression (Table 2). To verify whether *PXN* gene amplification could contribute to PXN overexpression in lung tumors, real-time PCR analysis was conducted to determine the extent of *PXN* amplification. Among 124 lung tumors, 17 tumors were detected with *PXN* amplification that was not associated with PXN mRNA and protein expression in lung tumors (PXN mRNA, $P = 0.433$; PXN protein, $P = 0.619$; Supplementary Tables 6 and 7). Moreover, *PXN* amplification was also not related with miR-218 expression ($P = 0.433$; Supplementary Table 8). Previously, there were no mutations for PXN identified in Taiwanese sample (0 of 70; ref. 4). We also used direct sequencing to detect mutations in exon 4 of the *PXN* gene. In lung cancer, in this exon has been shown to be the most frequently mutated site. No mutations in A127T in the *PXN* gene were found in the 124 lung tumors enrolled in this study (data not shown). These results suggest that PXN overexpression enhances tumor growth and metastasis in response to reduction of miR-218 rather than by gene mutation or gene amplification, at least in our studied population.

To determine whether PXN upregulation could affect certain important metastasis-related genes to cause tumor malignancy, the expression profiles of PXN-knockdown CL1-5 cells were analyzed by PCR array and compared with those of NC cells. Among the 2-fold and higher upregulated genes, CD44 molecule (CD44) showed the highest upregulation by PXN knockdown (12.0-fold), followed by serpin peptidase inhibitor, clade B (ovalbumin, SERPINB5; 8.32-fold), LY6/PLAUR domain containing 3 (LYPD3; 4.45-fold), TIMP metallopeptidase inhibitor 4 (TIMP4; 3.33-fold), chemokine (C-X-C motif), ligand 12 (CXCL12; 2.70-fold), platelet/endothelial cell adhesion molecule (PECAM1; 2.46-fold), and tumor-associated calcium signal transducer 1 (TACSTD1; 2.27-fold). On the other hand, chemokine (C-X-C motif) receptor 4 (CXCR4) and matrix metallopeptidase 7 (MMP7) were found to be downregulated (0.47-fold for CXCR4 and 0.49-fold for MMP7; Supplementary Table 9). CD44 expression has been shown to be relevant to tumor growth and metastasis in various human cancers including colon (36) and breast cancer (37), lymphomas (38), melanomas (39), and gastric (40) and lung carcinomas (41, 42). SERPINB5 (also known as Maspin) has been shown to be a tumor suppressor and may inhibit tumor growth and metastasis in breast cancer (43–45). These results suggest that tumor growth and metastasis enhanced by PXN overexpression may occur at least in part due to increased CD44 and SERPINB1 expression. However, the underlying mechanism of PXN overexpression on lung tumor growth and metastasis needs further exploration.

miR-218 is transcribed within the intronic region of the known tumor suppressor genes *SLIT2* (16, 22) and *SLIT3* (13). *SLIT2* has frequently been found to be inactivated in lung and breast tumors due to promoter hypermethylation (46), and *SLIT3* promoter hypermethylation in lung and breast cancer is rarer than *SLIT2* promoter hypermethylation (47). In this study, SLIT2 expression was significantly reduced in HPV16-infected TL-1 cells and E6-overexpressing TL-4 cells, and the alterations in SLIT2 expression was consistent with miR-218 suppression by E6 (Supplementary Fig. 1A). Methylation-specific PCR (MSP) analysis showed that promoter hypermethylation occurred in TL-1 and CL1-5 cells and that miR-218 and SLIT2 expression was restored when both cell types were treated with demethylated agent 5-aza-dC (Supplementary Fig. 1B and C). Therefore, miR-218 deregulation by E6 may partially occur via promoter hypermethylation of the *SLIT2* gene.

In summary, we provide the evidence to show that PXN upregulation in response to miR-218 reduction may enhance tumor growth and metastasis in lung cancer and that PXN and miR-218 may act as independent indicators of OS and RFS, respectively, in non–small cell lung cancer (NSCLC). We further found that the PXN level is useful for predicting the OS and RFS in NSCLC with HPV infection. Therefore, we recommend PXN as a potential target for therapeutics in NSCLC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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