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Original Article

Expression of poly(C)-binding protein 1 (PCBP1) in NSCLC as a negative regulator of EMT and its clinical value

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Abstract: Poly (C)-binding Protein 1 (PCBP1) is a 35 kDa protein involved in a number of biological processes. Recently, the research found that PCBP1 might be involved in epithelial-mesenchymal transition (EMT). However, the role of PCBP1 in non-small-cell lung cancer (NSCLC) metastasis needs further elucidation. The purpose of this study was to determine whether PCBP1 could serve as a biomarker for stratification and prediction of prognosis in NSCLC as a regulator of EMT formation. In this study, PCBP1 expression was evaluated by Western blot in 8 fresh lung cancer tissues and immunohistochemistry (IHC) on 145 paraffin-embedded slices. PCBP1 was highly expressed in non-metastatic NSCLC specimens and significantly correlated with lymph node status ($P < 0.001$), clinical stage ($P = 0.001$), vimentin expression ($P = 0.033$) and E-cadherin expression ($P = 0.042$). Our study showed that the low expression of PCBP1 was correlated with decreased expression of E-cadherin and elevated expression of vimentin, which were the markers of EMT. Besides, high expression of PCBP1 was correlated with better prognosis. These findings suggested that PCBP1 might play an important role in preventing the process of EMT in NSCLC, thus be a promising therapeutic target to inhibit NSCLC metastasis.

Keywords: Non-small-cell lung cancer, PCBP1, prognosis, EMT, metastasis

Introduction

Lung cancer is the leading cause of cancer-related death in the world [1]. Non-small-cell lung cancer (NSCLC) takes the main part of lung cancer. Although the rates of morbidity and mortality have decreased in NSCLC patients with operation recently, the prognosis of NSCLC remains unsatisfactory and the 5-year survival rate is limited to 15% [2], owing to the poor diagnostic rate in the early stage, high rates of recurrence and metastasis. However, metastasis remains the most poorly understood part of human malignancy, which is the major cause of cancer mortality. Diverse proteins and signaling pathways modulate cancer progression [3]. Thus a better understanding underlying molecular mechanisms of metastasis of NSCLC would significantly benefit the clinical outcome of NSCLC.

The epithelial-mesenchymal transition (EMT) was recently found to be essential for cancer metastasis and progression [4]. The EMT process enabled epithelial cells converse into mesenchymal cells, allowed their subsequent differentiation into multiple cell types during development and the initiation of metastasis and invasion, induced stem cell properties, inhibited apoptosis, and promoted carcinoma progression [5]. Epithelial cells must undergo both morphologic and functional changes for EMT via multiple signaling systems [6]. Various EMT-associated markers had been clarified for the biology of EMT, such as epithelial specific markers (E-cadherin and cytokeratin), mesenchymal specific markers (vimentin, fibronectin and N-cadherin), and transcription factors [7]. The process of EMT during cancer progression and metastasis consist of multiple steps. Firstly, cells detached from the primary tumor and invaded the surrounding tumor stroma. Subsequently, they entered into the vessel and reached new metastatic organs. Accordingly, molecular analyses based on EMT applied to

Table 1. PCBP1, vimentin and E-cadherin expression and clinicopathologic parameters in 145 NSCLC specimens

Parameters	Total	PCBP1 expression		*P*-value
		Low	High	
Age (year)				
< 60	53	35	18	0.482
≥ 60	92	55	37	
Gender				
Male	116	68	48	0.087
Female	29	22	7	
Smoking status				
Yes	53	28	25	0.109
No	92	62	30	
Histological type				
Adenocarcinoma	39	19	20	0.075
Squamous cell carcinoma	95	62	33	
Adenosquamous carcinoma	11	9	2	
Clinical stage				
I	62	28	34	0.001*
II	48	36	12	
III	35	26	9	
Histological differentiation				
Well	13	9	4	0.807
Mod	70	42	28	
Poor	62	39	23	
Lymph node status				
0	89	43	46	< 0.001*
> 0	56	47	9	
Vimentin expression				
Low	50	25	25	0.033*
High	95	65	30	
E-cadherin expression				
Low	74	52	22	0.042*
High	71	38	33	

Note. Statistical analyses were performed by the Pearson χ^2 test. *P < 0.05 was considered significant.

cancer progression. To identify the mechanism of EMT is of great significance.

EMT is highly controlled via autonomous oncogenic activation of signaling molecules without additional stimulation during cancer progression [6]. It was reported that various signaling pathways were involved in the regulation of EMT, such as TGF-β/signaling, Wnt signaling pathway, Notch signaling pathway. Many genes were transcriptionally altered in EMT involved in mesenchymal differentiation, cell adhesion, cell migration, and invasion [8]. Recently, we identified Poly(C)-binding Protein 1 (PCBP1) as a regulator of EMT, which belonged to the family of PCBPs. In mammalian cells, these PCBPs belonged to one of two subsets: hnRNP K/J, or the alpha-complex proteins (PCBP1-4) [9]. PCBPs were known to be involved in various biological processes, such as: transcriptional activator, regulator of RNA splicing, transportation process, function of RNA molecules, and translational repressor, through their poly (C)-binding ability [10-12]. PCBPs functioned as regulators of transcription by binding to specific elements on gene promoters in eukaryotic cells [13]. The diverse functions of PCBPs suggested that they acted both in the cytoplasm (translation) and in the nucleus (transcription and splicing) [14].

PCBP1, a member of the PCBPs family, was especially investigated. PCBP1 participated in regulations of the androgen receptor gene [15], eIF4E gene [16], the MOR gene [17, 18] and so on. PCBP1, widely expressed in many human tissues, could act at multiple levels during gene expression. Phosphorylation of PCBP1 induced by phosphorylation of p21-activated kinase 1 could increase its nuclear retention and enhanced PCBP1-dependent splicing events [16]. Knockdown of PCBP1 caused upregulation of PRL-3 protein levels [3], activation of AKT [19], and promotion of tumorigenesis. It was reported that PCBP1 had a widely function in tumors, like hepatocellular carcinoma [20], breast cancer, prostate cancer [21, 15] and cervical cancer [22]. Overexpression of PCBP1 in HepG2 cells dramatically inhibited tumor invasion [20] and decreased AR protein in the prostate cancer LNCaP cell line. PCBP1 was reported to regulate TGF-β mediated EMT in breast epithelial cells and human lung cancer cell line A549 [23], and could suppress metastasis of colon cancer cell [3].

Although PCBP1 is involved in various biological events, the clinical significance of PCBP1 contributes to NSCLC remains largely unknown. It is tempting for us to speculate that PCBP1 may involve in NSCLC metastasis. In this study, we aimed to perform a comprehensive analysis of PCBP1 in surgically resected samples with

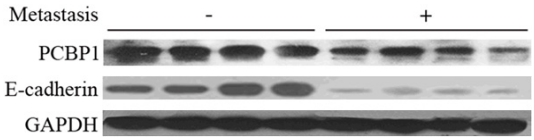

Figure 1. Expressions of PCBP1 and E-cadherin in 8 NSCLC tissue specimens with or without lymph node metastasis. A representative Western blot image showed that the PCBP1 expression was significantly lower in metastatic tissues than in non-metastatic ones. E-cadherin was used as an epithilial marker. GAPDH was used as a control for protein load and integrity.

NSCLC, in order to receive more detailed insights into the complicated interactions of each individual variances with patients' outcome. We found that, PCBP1 was lowly expressed in highly lymph node metastasis NSCLC tissues, and related to NSCLC histological differentiation, clinical stage and lymph node. Our research implicated that PCBP1 might function as a negative regulator in NSCLC via EMT formation.

Materials and methods

Patients and tissue samples

All human tissues were collected using protocols approved by the Ethics Committee of Affiliated Hospital of Nantong University. The NSCLC fresh samples were obtained immediately after surgical removal, and stored at -80°C. For histological examination, tissues were obtained from 145 patients who underwent lung resection without preoperative systemic chemotherapy at the Surgery Department of the Affiliated Hospital of Nantong University from 2005 to 2009. All the specimens were fixed in formalin and embedded in paraffin. After obtaining informed consent, patients were interviewed to obtain information on demographic characteristics, and clinical data were collected. The follow-up time was 5 years. The main clinical and pathologic variables were shown in **Table 1**. 116 patients were men while 29 were women, and their average age was 63 years (range, 39-83). Tumors were classified as well (grade I; n = 13), moderately (grade II; n = 70), or poorly (grade III; n = 62) differentiated.

Western blot and antibodies

Tissue protein was promptly homogenized in a homogenization buffer containing 50 mM Tirs-HCl, PH7.5, 0.1% NP-40, 60 mM β-glycerophosphate, 150 mM NaCl, 5 mM EDTA, 0.1 mM NaF, 0.1 mM sodium orthovanadate, and complete protease inhibitor cocktail (Roche Diagnostics), and then centrifuged at 13000 g for 20 min to collect the supernatant. Protein concentrations were determined with a Bio-Rad protein assay (BioRad, Hercules, CA, USA). Proteins were separated with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride filter (PVDF) membranes (Millipore, Bedford, MA). The membranes were blocked with 5% fat-free milk in TBST (20 mM Tris, 150 mM NaCl, 0.05% Tween-20) for 2 h at room temperature, and then the filters were washed with TBST thrice and incubated overnight with polyclonal antibody against using the primary antibodies described later and horseradish peroxidase-linked IgG as the secondary antibodies. Immunoreactive bands were visualized by chemiluminescence detection system (Pierce). After the chemiluminescence was exposed to X-ray films, the films were scanned with a Molecular Dynamics densitometer (Imaging Technology, Ontario, Canada). The band density was measured with a computer-assisted image-analysis system (Adobe Systems, San Jose, CA) and normalized against GAPDH level. The experiments were repeated for three times.

The antibodies used for Western blot in this study included: mouse anti-human PCBP1 antibody, rabbit anti-human E-cadherin antibody, and rabbit anti-human GAPDH antibody, all the antibodies were obtained from Santa Cruz Biotechnology, USA.

Immunohistochemistry (IHC)

Tissue sections were 4 μm thick, dewaxed in xylene and rehydrated in graded ethanols. Endogenous peroxidase activity was blocked by immersion in 3% methanolic peroxide for 10 min. And then, the sections were processed in 10 mmol/L citrate buffer (pH 6.0) and heated to 121°C in an autoclave for 20 minutes to retrieve the antigen. After rinsing in phosphate-buffered saline (PBS) (pH7.2), the sections were then incubated 2 h at room temperature with mouse anti-human PCBP1 antibody (diluted 1:500, Santa Cruz Biotechnology, USA), rabbit anti-human E-cadherin antibody (diluted 1:500, Santa Cruz Biotechnology, USA), rabbit anti-human vimentin antibody (diluted 1:500, Santa Cruz Biotechnology, USA). Negative control slides were processed in parallel using a

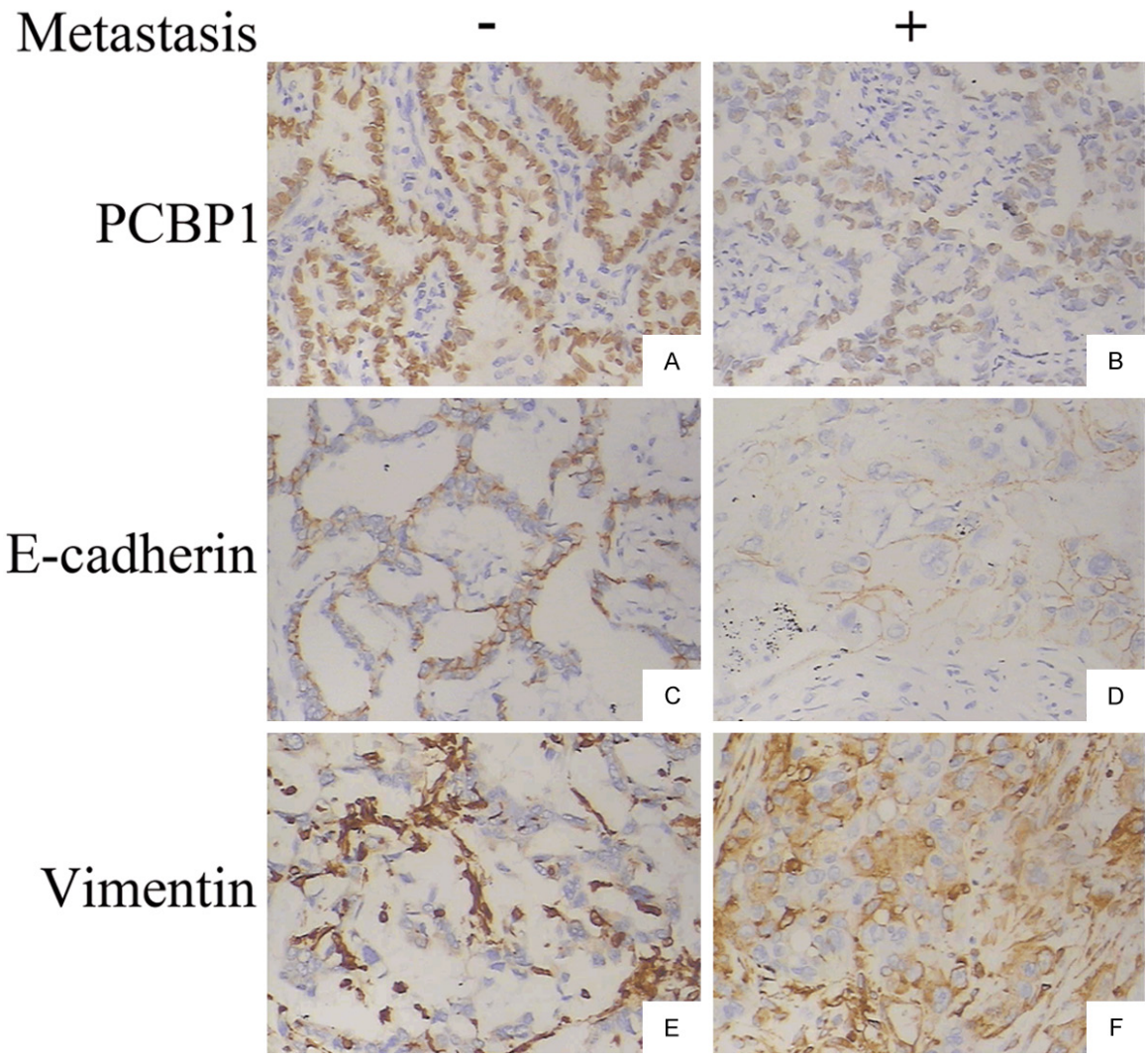

Figure 2. Immunohistochemical analysis of PCBP1, E-cadherin and vimentin expression in NSCLC tissues. A-F: Paraffin-embedded tissue sections were stained with antibodies of PCBP1, E-cadherin and vimentin, and counterstained with hematoxylin. A and C: Both high PCBP1 and E-cadherin expression in NSCLC specimens with no lymph node metastasis. B and D: Both low PCBP1 and E-cadherin expression in NSCLC specimens with lymph node metastasis. E: Low vimentin expression in NSCLC specimens with no lymph node metastasis. F: High vimentin expression in NSCLC specimens with lymph node metastasis. The experiment details were described in Materials and Methods. Magnification × 400. All the results were repeated at least three times.

nonspecific immunoglobulin IgG (Sigma Chemical Co, St. Louis, MO) at the same concentration as the primary antibody. After washed in PBS, all slides were processed using the peroxidase-antiperoxidase method (DAKO, Hamburg, Germany), according to the manufacturer's instructions. All slides were counterstained with hematoxylin, dehydrated, and mounted in resin mount.

Immunohistochemical evaluation

All the immunostained sections were evaluated in a blinded manner without knowledge of the clinical and pathological parameters of the NSCLC patients. PCBP1 positive cells were counted by monitoring at least 1000 cells from at least 5 randomly selected fields. To allow univariate and multivariate analyses, the intensity of immunostaining in each tumor section was assessed as strong (3), moderate (2), weak or negative (1); semiquantitatively using the following scale: < 5% of cells (0), 5-25% (1), 26-50% (2), 50-75% (3), and > 75% (4) of cells. Then we multiplied the two scores, and classified them into two groups: score of 1 (0-6, low expression), score of 2 (7-12, high expression). The cutoff of E-cadherin expression was defined

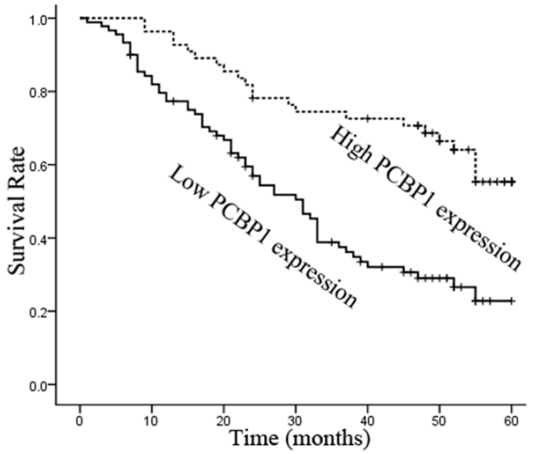

Figure 3. Correlation between survival and the expression of PCBP1. Based on the mean percentage of PCBP1-positive cells, patients were divided into low PCBP1 expressers (scores 0-6) and high PCBP1 expressers (scores 7-12). Patients in the high PCBP1 expresser group had significantly better overall survival ($P < 0.05$).

as 70%. Tumor cells more than > 70% showed membranous staining in the entire tumors was considered as positive. While, Vimentin expression was often found in tumor cytoplasm, and more than 20% of tumor cells showed that cytoplasm staining was positive. In half of the samples, staining was repeated three times to avoid possible technical errors, but similar results were obtained in these samples. The above procedures of evaluation were performed by three independent pathologists with a multihead microscope, and a consensus was achieved.

Statistical analysis

All computations were carried out using the SPSS 17.0 statistical program. All values were expressed as means ± SEM. The PCBP1, E-cadherin and Vimentin expression and clinicopathological features were analyzed using the χ^2 test. Kaplan-Meier curves were constructed, and the log-rank test was performed for analysis of survival data. Multivariate analysis was performed by Cox's proportional hazards model, and the risk ratio and its 95% confidence interval were recorded for every marker. $P < 0.05$ was required for statistical significance. Values were responsible for at least three independent reactions.

Results

PCBP1 was lowly-expressed in highly lymph node metastasis NSCLC tissues

To evaluate the expression and clinical significance of PCBP1 in NSCLC metastasis and further characterize the relations between PCBP1 and EMT markers like E-cadherin and vimentin, we performed Western blot in 8 metastatic and non metastatic fresh NSCLC tissues. As shown in **Figure 1**, the expression of PCBP1 in NSCIC was similar to that of E-cadherin, both of which had lower expression in metastatic cancer tissues than that in non-metastatic tissues.

To confirm the role of PCBP1 in NSCLC, we investigated the expression of PCBP1, vimentin and E-cadherin using IHC. Representative examples of reactivity for PCBP1, vimentin and E-cadherin were shown in **Figure 2**. As the result, PCBP1 was down-regulated in metastatic NSCLC specimens, and might be an important target for NSCLC EMT formation.

Correlation of PCBP1 expression with clinicopathologic features in NSCLC

To further explore the physiological and pathological relationship between the expression of PCBP1 and EMT markers in NSCLC, the presence of PCBP1 in varies pathological types were summarized in **Table 1**. It was found that PCBP1 expression was significantly associated with lymph node status ($P < 0.001$), clinical stage ($P = 0.001$), vimentin expression ($P = 0.033$) and E-cadherin expression ($P = 0.042$). But there was no correlation between PCBP1 expression and other prognostic factors such as age, gender, smoking status, histological type and histological differentiation. In addition, the expression of PCBP1 was low in metastatic carcinoma cells with the same tendency of E-cadherin, while the opposite tend of vimentin (**Table 1**).

PCBP1 was significantly associated with the survival of NSCLC patients

Survival information was available for all patients with follow-up data. To estimate the prognostic significance of PCBP1, we performed the Kaplan-Meier analysis, patients with PCBP1-positive tumor were significantly correlated with better overall survival ($P <$

Table 2. Contribution of various potential prognostic factors to survival by Cox regression analysis in 145 NSCLC specimens

	Hazard ratio	95.0% Confidence interval	P
Age	0.973	0.581-1.630	0.917
Gender	0.752	0.428-1.319	0.320
Smoking status	0.763	0.452-1.286	0.310
Histological type	0.935	0.617-1.416	0.752
Clinical stage	1.565	1.146-2.139	0.005*
Histological differentiation	1.063	0.756-1.494	0.725
Lymph node status	1.139	0.619-2.098	0.675
PCBP1 expression	0.549	0.303-0.994	0.048*
Vimentin expression	1.437	0.777-2.657	0.248
E-cadherin expression	0.602	0.379-0.958	0.032*

Note. Statistical analyses were performed by the Cox regression analysis. *$P < 0.05$ was considered significant.

0.001; **Figure 3**). Multivariate analysis using the Cox's proportional hazards model showed that PCBP1 ($P = 0.048$), clinical stage ($P = 0.005$) and E-cadherin ($P = 0.032$), significantly influenced the survival of NSCLC patients (**Table 2**). To conclude, multivariate analysis using the Cox's proportional hazards model indicated that PCBP1 ($P = 0.048$; **Table 2**) was a prognostic factor for patients' overall survival.

Discussion

NSCLC shows a high percentage of recurrence and metastasis, leading to high mortality. To develop novel treatments, it is imperative to address the factors underlying tumorigenesis, invasion and metastasis. In this study, we identified PCBP1 to be a protein lowly-expressed in NSCLC specimens with metastasis. Differential expression of PCBP1 was detected between primary cancer tissues with or without lymph nodes metastasis, which was correlated with clinical stage, lymph node status, EMT markers like E-cadherin and vimentin. PCBP1 might function as an important player in NSCLC metastasis and progression. PCBP1 expression was significantly higher in NSCLC tissues with no lymph nodes metastasis compared with the tissues with lymph nodes metastasis. The Cox's proportional hazards model indicated that high expression of PCBP1 might be a protector for the patients. Taken together, these findings supported the hypothesis that PCBP1 could be an important regulator of EMT in NSCLC and a prognostic factor for patients.

EMT is an essential step in embryogenesis, and cancer progression, via which tissue epithelial cancers invade and metastasis. During this transition, the epithelial phenotype, characterized by strong cell-cell junctions and polarity, is replaced by a mesenchymal phenotype, reduced cell-cell and cell-matrix interactions, and increased motility and invasiveness [24]. However, the demonstration of this process in human cancer remained controversial. Thus, to explore the proteins functioned in the EMT of NSCLC that leading to metastasis is of great importance. Aberrant regulation or genetic mutations in metastasis-related genes were found in most NSCLC specimens. Dysregulation of various oncogenic and tumor-suppressing signaling might be key factors for normal bronchus epithelial cell oncogenesis.

PCBP1, approximately 38 kDa in size, has been shown to be involved in a wide range of biological processes including transcription, mRNA stability and translation, as well as protein-protein interactions [10]. Loss of PCBP1 expression was previously correlated to telomeric shortening, oncogenic transformation [25]. PCBP1 was negatively-regulated in diverse human cancers, played an important role in spreading initiation centers at onset of tumor invasion and metastasis [26]. For example, PCBP1 was an important regulator of TGF-β signaling pathway in gall bladder cancer cell line GBC-SD [25]. Furthermore, knockdown of PCBP1 in the prostate cancer LNCaP cell line also increased AR protein [15], and contributed to the formation of a metastatic phenotype in human hepatic tumors [27]. However, some reports indicated that phosphorylated PCBP1 could induce EMT and was involved in the invasiveness and metastasis.

The link between PCBP1 and cancer-related signaling was discovered recently, while TGF-β signaling was mostly identified. It was reported that overexpression of PCBP1 in A549 cells inhibited ILEI translation, while ILEI driven epithelial to mesenchymal transition and metastatic progression in A549 cells [23]. To date, there is no study about the clinical significance

of PCBP1 in NSCLC, and the present study is the first to analyze the possible associations with clinicopathological parameters as well as patients' prognosis. In the study, we identified that PCBP1 was a candidate of NSCLC EMT regulator, which might be a major cause for its low-expression in NSCLC specimens. However, our results indicated that PCBP1 expression might be involved in the EMT of NSCLC, highlighting PCBP1 functioned as a potential therapy target for the metastasis and prognosis of this malignancy. In this study, we found PCBP1 had the same tendency with E-cadherin. E-cadherin was a key component of adherens junctions, structures that played crucial roles in the maintenance of epithelial integrity. Loss of E-cadherin function through genetic or epigenetic mechanisms was implicated in the progression and metastasis of numerous malignancies [24]. While, survival curve revealed PCBP1 to be an indicator of NSCLC patients' prognosis. Thus, our findings supported the notion that PCBP1 as a negative regulator of EMT might help regulating the metastasis and progression of NSCLC.

In summary, we suggested that expression of PCBP1 exerted an inhibitory effect on the invasive properties of complete moles, and this inhibitory effect was reduced when PCBP1 was downregulated in NSCLC. Our preliminary survival analysis indicated that high PCBP1 expression might be associated with better overall survival, but this early finding need to be confirmed with a larger group of patients. From these data, we could expect that up-regulation of PCBP1 might potentially be an effective therapeutic approach for the inhibition of cell migration and invasion of NSCLC and other human cancers. However, further studies are necessary to elucidate the molecular mechanisms of PCBP1 in NSCLC pathogenesis.

Disclosure of conflict of interest

None.

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