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PPM1H is an independent prognostic biomarker of non-small-cell lung cancer

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Protein phosphatase 1H (PPM1H) is the metal-dependent protein phosphatase, however, its role in tumorigenesis and tumor progression remains controversial. Non-small-cell lung cancer (NSCLC) is the most common histological type of lung cancer but the expression and clinical significance of PPM1H in NSCLC is unknown. In our study, we detected the mRNA of PPM1H in 25 pairs of NSCLC tissues and their corresponding adjacent tissues with qRT-PCR. Moreover, we investigated PPM1H expression in 474 NSCLC tissues and divided them into subgroups with low and high PPM1H. We further evaluated its correlation with the clinicopathological factors. The correlation between PPM1H and other biomarkers involved in tumor progression including chromosome segregation 1-like protein (CSE1L), p53, and Ki67 was also estimated. In addition, the prognostic significance of PPM1H was investigated by univariate and multivariate analyses. The mRNA levels of PPM1H in NSCLCs were significantly higher than those in tumor-adjacent tissues. Patients with low and high PPM1H expression accounted for 54.64% (259/474) and 45.36% (215/474) respectively in all the NSCLCs. PPM1H expression (p=0.012), patients' sex (p=0.009), tumor size (p<0.001), histological grade (p=0.026), T stage (p=0.002), N stage (p<0.001), M stage (p=0.011), and TNM stage (p<0.001) were all associated with the poor prognosis. With multivariate analysis, PPM1H was determined as an independent prognostic factor of NSCLC (HR=1.42, 95% CI=1.14–1.75, p=0.001). Moreover, high PPM1H was significantly with high Ki67 (p=0.022), indicating the oncogenic role of PPM1H. PPM1H is an independent prognostic factor indicating an unfavorable prognosis of NSCLC. Our results indicated that PPM1H was an important supplement of NSCLC molecular profile and detecting PPM1H may help recognize the high-risk patients for further treatment.

Key words: PPM1H, prognosis, non-small-cell lung cancer, biomarker

Lung cancer is the most common cancer type worldwide, accounting for approximately 18.1 million new cases, and resulting in the most cancer-related deaths [1]. Lung cancer mainly consists of two histological types, small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). NSCLC accounts for about 85% of all lung cancers and it can be further divided into three major histological subtypes: large cell carcinoma (LCC), squamous cell lung cancer (SCC), and adenocarcinoma (AC) [2]. There are several important breakthroughs in NSCLC treatment, such as the application of EGFR inhibitor, which greatly improved the overall survival (OS) time of patients. However, the prognosis of lung cancer remains very poor, with a 5-year OS rate of about 18.1% [3]. The new prognostic biomarkers and new target therapies are still required to improve the overcome of patients.

Protein phosphatase 1H (PPM1H) is a member of the PPM family (the metal-dependent protein phosphatases, also called PP2C) of serine/threonine protein phosphatases [4]. PPM family dephosphorylates the substrates in the involvement of metal cations especially Mn^{2+} and Mg^{2+} [5, 6]. PPM1H was originally identified as a negative regulator of neurite outgrowth, but emerging evidence suggested it was involved in tumor progression, including colon cancer and glioma [7–9]. However, the studies of PPM1H are rare and the functions of PPM1H in cancer development had not got the consensus. A previous study showed that PPM1H promoted the proliferation of colon cancer [9], while contrary proofs suggested that low PPM1H of tumor tissues indicated poor prognosis in colorectal cancer [10]. So here we investigated the expression and clinical significances of PPM1H in lung cancer.

In our study, we detected the mRNA of PPM1H in 25 pairs of NSCLC tissues and their corresponding adjacent tissues with qRT-PCR. Moreover, we investigated PPM1H expression in 474 NSCLC tissues and divided them into subgroups with low and high PPM1H. We further evaluated its correlation with the clinicopathological factors. In addition, the prognostic significance of PPM1H was investigated by univariate and multivariate analyses.

Patients and methods

Patients and follow-up. Between 2010 and 2018, a total of 849 patients diagnosed, as NSCLC who underwent radical surgery in YIDU Central Hospital and Yantaishan Hospital, constituted the test cohort. The test cohort was comprised of 502 male and 347 female patients. From the test cohort, 474 patients were selected into the validation cohort if they followed the criteria as: 1) survival time more than 3 months, 2) enough specimens for immunohistochemistry (IHC), 3) available follow-up. There were 288 ACs and 186 SCCs in the validation cohort, which consisted of 259 male patients and 215 female patients, with an average follow-up as 30.22 months (Table 1). No patients received pre-operational adjuvant therapies including chemotherapy and radiotherapy, and all the patients received standard adjuvant therapies based on the NSCLC treatment criteria. The TNM stage system was referred to the 8th AJCC/UICC system.

Ethical statement. All the paraffin-embedded specimens were obtained from the Department of Pathology in YIDU Central Hospital and Yantaishan Hospital with the prior consent of patients. The study was approved and supervised by the Ethics Board of YIDU Central Hospital and Yantaishan Hospital. All procedures performed in studies involving human participants were in accordance with the ethical standards of YIDU Central Hospital, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

RNA extraction and qRT-PCR. A total of 25 fresh pairs of NSCLC tissues and their tumor-adjacent tissues were obtained during surgery without interfering with the pathological diagnosis. qRT-PCR was performed to compare PPM1H mRNA between NSCLCs and adjacent tissues. Total RNAs were extracted with TRIzol according to the manual. The cDNA was reversely transcribed with a cDNA synthesis kit (Applied Biosystems). SYBR green method was used to quantify cDNA reverse transcription with a qPCR machine (7900HT, Applied Biosystems). The results were analyzed with the $2^{\Delta\Delta CT}$ method with GAPDH as an internal control. The primers of PPM1H were as follows: forward: ATATGGAGAAGGCAAGAAGG, reverse: TCATATCTGGAGAGATCGTAG.

Immunohistochemistry. The expression of PPM1H was detected with IHC using the streptavidin-biotin immunoperoxidase method. In brief, after deparaffinization and rehydration with xylene and ethanol, the specimens were soaked in 3% H_2O_2 for 30 min for the inactivation of endogenous peroxidase and then incubated in citrate buffer (pH 6.0) for optimal antigen retrieval. After incubation in 1% bovine serum albumin for 1 h to block unspecific antigen binding, the slides were rinsed by phosphate buffer saline and then incubated in the primary antibody (Invitrogen, PA5-26102) at 1:200 dilution at 4 °C overnight. After that, the corresponding secondary antibody (Santa Cruz) was applied to incubate the specimens for 1 h at room temperature, followed by the application of the streptavidin-peroxidase complex (Beyotime, Shanghai, China) and 3,3'-diaminobenzidine solution (Beyotime) for final visualization.

The following antibodies were used: PPM1H (Invitrogen, PA5-26102, 1:100), p53 (Santa Cruz Biotechnology, DO-1, 1:200), CSE1L (Proteintech Group, 22219-1-AP, 1:100), and Ki67 (Invitrogen, MA5-14520).

Semi-quantification of IHC. The results of IHC were semi-quantified by the IHC score by two senior pathologists who were unaware of the clinical data of patients. In brief, the scores for staining intensity were defined as 0 for negative staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining; the scores for the percentage of positive cells were set as 0 for 10% positive cells, 1 for 10–25% positive cells, 2 for 25–50% positive cells, 3 for 50–75% positive cells and 4 for >75% positive cells. The total IHC score was set as the multiplication of staining intensity and positive cells, ranging between 0 and 12. The validation cohort was divided into the patients with low or high expression of PPM1H by the cut-off, which was set by the receiver operating characteristic (ROC) curve. The point which had the highest sum of sensitivity and specificity in the ROC curve was set as the cut-off, which was 5.0 for PPM1H and 3.5 for CSE1L in our study.

Statistical analysis. All data were analyzed with the software SPSS22.0 (IBM, Chicago, IL, USA). The correlations between PPM1H expression and the clinicopathological factors were calculated with the χ^2 test. The prognostic factors were determined by the univariate analysis. OS curves were displayed with the Kaplan-Meier method and the statistical significance was calculated with the log-rank test. The multivariate analysis with the Cox proportional hazards regression model was performed to identify the independent prognostic factors. A p-value <0.05 was defined as statistically significant.

Results

The expression of PPM1H in NSCLCs and tumor-adjacent tissues. Firstly, the expressions of PPM1H in 25 pairs of NSCLC tissues and their corresponding adjacent tissues were detected with qRT-PCR. The mRNA levels of PPM1H in NSCLCs were significantly higher than those in tumor-adjacent tissues (Figure 1A), indicating that aberrant PPM1H upregulation may contribute to the tumorigenesis

of NSCLC. Moreover, the expression of PPM1H in paraffin-embedded NSCLCs was detected with IHC. The total 474 patients comprised 288 ACs and 186 SCCs. The expression of PPM1H was mainly observed in the nuclei of NSCLC. The patients were divided into subgroups with low and high PPM1H expression, which accounted for 54.64% (259/474) and 45.36% (215/474), respectively (Figure 1B; Table 1).

Correlations between PPM1H and the clinical variables. The correlations between PPM1H and the clinicopathological variables were further evaluated with the χ^2 test (Table 2). The enrolled clinicopathological variables included patients' sex, age, tumor diameter, histological type, histological grade, T/N/M stage, and TNM stage. PPM1H had no significant correlations with these factors, which showed that the baseline of our cohort was balanced and that a further survival analysis could be performed.

Prognostic factors were determined by univariate analysis. The univariate analysis was performed to determine the prognostic factors of NSCLC (Table 3). The Kaplan-Meier method was applied to show the OS curve and the log-rank test was used to calculate the statistical difference. In our study, PPM1H was an independent prognostic factor of NSCLC. The 5-year OS rates of low and high expression of PPM1H were 24.9% and 17.7%, respectively (Figure 2A). High expression of PPM1H indicated the unfavorable prognosis of NSCLC (p=0.012). Despite the PPM1H expression, patients' sex (Figure 2B), tumor size (Figure 2C), histological grade (Figure 2D), T stage (Figure 2E), N stage (Figure 2F), M stage (Figure 2G), and TNM stage (Figure 2H) were all associated with the poor prognosis. Male sex (p=0.009), large tumor size (>5 cm; p<0.001), high histological grade (p=0.026), advanced T (p<0.001) and N stage (p<0.001), positive metastasis (p=0.011), and advanced TNM stage (p<0.001) all predicted the poor prognosis of NSCLC.

The best cut-off of IHC score is still controversial, although most studies used the ROC curve to determine the cut-off, which was also applied in our study. To further verify our results, we used the average IHC score of PPM1H as the

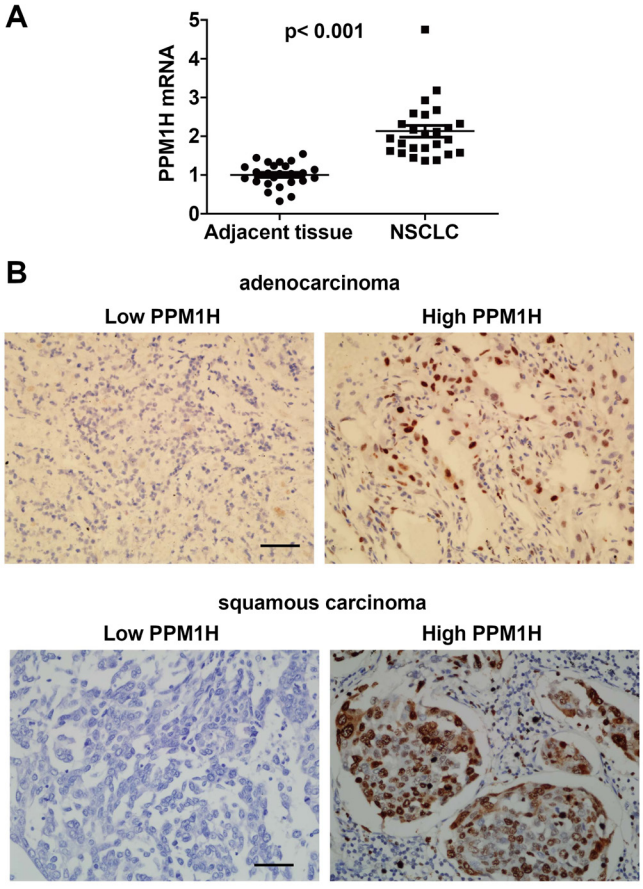


Figure 1. The expression of PPM1H in NSCLC and NSCLC-adjacent tissues. A) The mRNA levels of PPM1H in 25 pairs of NSCLC tissues and their tumor-adjacent tissues were detected with qRT-PCR. The differences between subgroups were compared with paired t-test. B) The expression of PPM1H in 474 cases of NSCLCs was detected with IHC, dividing the patients into low and high PPM1H expression. Scale bar: 100 μm

Table 1. Baseline of the test and validation cohort.

Factors	Test cohort		Validation cohort	
	n	%	n	%
Sex				
Male	502	59.13	259	54.64
Female	347	40.87	215	45.36
Age				
<60	275	32.39	168	35.44
≥60	574	67.61	306	64.56
Tumor diameter				
≤5cm	533	62.78	268	56.54
>5cm	316	37.22	206	43.46
Histological type				
Adenocarcinoma	453	53.36	288	60.76
Squamous carcinoma	396	46.64	186	39.24
Histological grade				
I+II	523	61.60	301	63.50
III	326	38.40	173	36.50
T stage				
I+II	567	66.78	332	70.04
III+IV	282	33.22	142	29.96
N stage				
N0	455	53.59	227	47.89
N1–N3	394	46.41	247	52.11
Metastasis				
No	802	94.46	453	95.57
Yes	47	5.54	21	4.43
Operation procedures				
Pneumonectomy	97	11.43	51	10.76
Lobectomy	545	64.19	292	61.60
Wedge resection	207	24.38	131	27.64
TNM stage				
I+II	465	54.77	245	51.69
III+IV	384	45.23	229	48.31

Table 2. Correlation between PPM1H expression and the clinicopathological factors.

Factors	PPM1H		
	Low	High	p-value*
Sex			
Male	130	129	0.233
Female	129	86	
Age			
<60	92	76	0.969
≥60	167	139	
Tumor size			
≤5 cm	148	120	0.771
>5 cm	111	95	
Histological type			
Adenocarcinoma	158	130	0.925
Squamous carcinoma	101	85	
Histological grade			
I+II	163	138	0.848
III	96	77	
T stage			
I+II	183	149	0.763
III+IV	76	66	
N stage			
N0	122	105	0.707
N1–N3	137	110	
Metastasis			
No	245	208	0.252
Yes	14	7	
Operation procedures			
Pneumonectomy	29	22	0.796
Lobectomy	156	136	
Wedge resection	74	57	
TNM stage			
I+II	138	107	0.461
III+IV	121	108	

Note: *χ^2 test

Table 3. Prognostic factors were confirmed by the univariate analysis.

Factors	Univariate analysis	
	5-year OS rate	p-value*
Sex		
Male	18.9	0.009
Female	26.0	
Age		
<60	22.4	0.093
≥60	20.9	
Tumor size		
≤5 cm	25.6	<0.001
>5 cm	18.1	
Histological type		
Adenocarcinoma	23.9	0.740
Squamous carcinoma	18.3	
Histological grade		
I+II	24.5	0.026
III	16.2	
T stage		
I+II	24.6	0.002
III+IV	18.1	
N stage		
N0	36.1	<0.001
N1–N3	8.9	
Metastasis		
No	22.5	0.011
Yes	0.0	
Operation procedures		
Pneumonectomy	32.7	0.864
Lobectomy	26.0	
Wedge resection	35.1	
TNM stage		
I	52.7	<0.001
II	26.0	
III	5.1	
IV	0.0	
PPM1H		
Low	24.9	0.012
High	17.7	

Note: *log-rank test

cut-off and analyzed the prognostic significance by univariate analysis (Supplementary Figure S1). The average IHC score of PPM1H divided the 474 patients into 237 patients with low PPM1H and 237 patients with high PPM1H. With the log-rank test, we showed that this classification method also supported that PPM1H was a poor prognostic biomarker (p=0.012), which was consistent with the results of the ROC-defined cut-off.

PPM1H was an independent prognostic factor of NSCLC. The prognostic factors in univariate analyses were selected into the Cox-regression model for multivariate analysis to identify the independent prognostic factors except for the TNM stage. In the enrolled factors, PPM1H was determined as an independent prognostic factor of NSCLC (HR=1.42, 95% CI=1.14–1.75, p=0.001; Table 4). In addition, the male sex (p=0.016), large tumor size (p=0.005), advanced N stage (p<0.001), and positive metastasis (p=0.001) were all verified as the independent prognostic factors of NSCLC. Our results suggested that PPM1H was an effective prognostic biomarker of NSCLC and was able to predict poor prognosis.

PPM1H was significantly associated with high Ki67 in NSCLC. In Table 1, we observed that PPM1H was significantly associated with T stage and lymphatic invasion, showing that PPM1H was a key effector in NSCLC progression. A previous study indicated that PPM1H promoted the proliferation of colon cancer by catalyzing CSE1L, a proliferation and apoptosis-related protein [7]. So, we further detected the expression of CSE1L and other well-known progression-related biomarkers, such as p53 and Ki67, and evaluated their correlations with PPM1H expression. The expressions

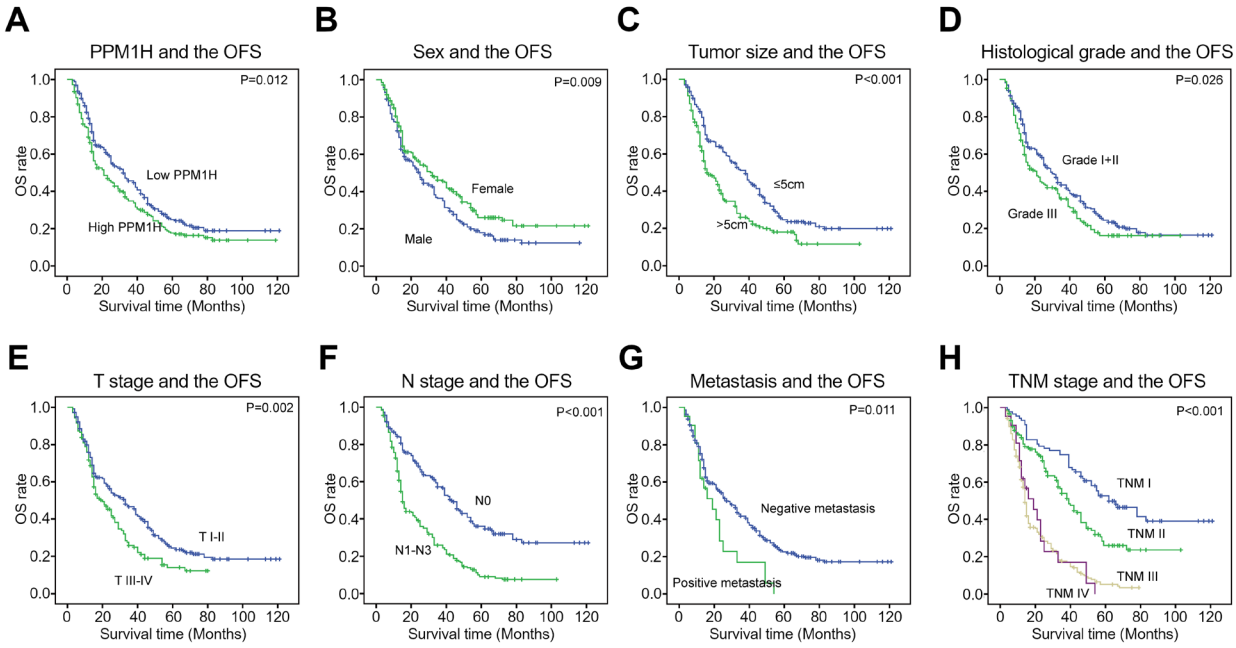


Figure 2. The correlations between PPM1H, sex, tumor size, histological grade, T stage, N stage, metastasis, TNM stage, and OS rates. The OS curves were displayed with the Kaplan-Meier method based on the PPM1H expression, patients' sex, tumor size, histological grade, T stage, N stage, metastasis, and TNM stage. PPM1H expression, patients' sex, tumor size, histological grade, T stage, N stage, M stage, and TNM stage were all associated with the poor prognosis. The statistical difference between OS curves was analyzed with the log-rank test.

of CSE1L, p53, and Ki67 in all the 474 NSCLCs were detected with IHC (Figure 3A), which divided the cohort into subsets with low or high expression. By χ^2 test, we demonstrated that PPM1H expression was not correlated with CSE1L or p53 expression, but was associated with high Ki67 expression (p=0.022; Figure 3B). These results supported that PPM1H was involved in NSCLC progression, which may require Ki67 participation.

Discussion

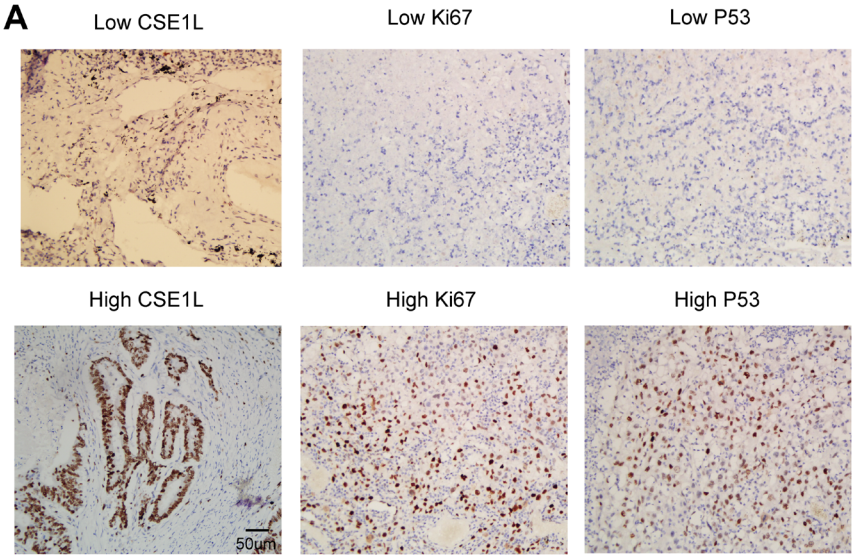
There were two breakthroughs in the treatment of NSCLC. The first breakthrough was the application of tyrosine kinase inhibitor (TKI) therapies, and the second breakthrough was the prospective molecular profiling by high-throughput sequencing [11]. Before the administration of EGFR inhibitor therapy, the OS time of most patients with NSCLC is less than 1 year, even treated with platinum-based combination chemotherapy [11]. Moreover, the molecular profiling by high-throughput sequencing revealed that almost two-thirds of patients with NSCLC harbor an oncogenic driver mutation, and half of them have a therapeutically targetable lesion [11], providing essential evidence for the individual therapy [12]. Both breakthroughs unveil new insights to the treatment of NSCLC and help to improve the OS time of patients. However, the prognosis of patients with NSCLC remains unsatisfactory due to the high heterogeneity of lung

Table 4. Independent prognostic factors were identified by the multivariate analysis.

Factors	Multivariate analysis		
	HR	95% CI	p-value*
Sex			
Female	1		
Male	1.31	1.05–1.64	0.016
Tumor size			
≤5 cm	1		
>5 cm	1.41	1.11–1.80	0.005
Histological grade			
I+II	1		
III	1.13	0.91–1.41	0.273
T stage			
I+II	1		
III+IV	1.17	0.91–1.51	0.219
N stage			
N0	1		
N1–N3	2.28	1.82–2.85	<0.001
Metastasis			
No	1		
Yes	2.180	1.36–3.49	0.001
PPM1H			
Low	1		
High	1.42	1.14–1.75	0.001

Note: *cox-regression model

A



B

Factors	number	PPM1H Low	PPM1H High	P*
CSE1L				
low	329	184	145	0.424
high	145	75	70	
Ki67				
<20%	300	176	124	0.022
≥ 20%	174	83	91	
p53				
<70%	277	154	123	0.641
≥ 0%	197	105	92	

* Chi-sqaure test

Figure 3. The correlations between PPM1H expression and CSE1L expression, p53 and Ki67 percentage. A) The expression of CSE1L, p53, and Ki67 was detected with IHC. Low and high expression of CSE1L, p53, and Ki67 were displayed. B) The correlations between PPM1H expression and CSE1L expression, p53, and Ki67 percentage were analyzed with the χ^2 test.

cancer, requiring the discovery of new therapies. Important target therapies rely on the identification of new biomarkers. For example, the demonstration of EGFR mutation functions in NSCLC directly leads to the development of TKI therapy. Here we demonstrated that PPM1H was an independent prognostic factor of NSCLC by a large cohort. The well-accepted prognostic factors verified by previous studies, such as T/N/M and TNM stage, all had significant correlations with prognosis, indicating the validity of our validation cohort. Our results suggested that PPM1H detection could predict prognosis and may be a potential drug target in NSCLC. The detection of PPM1H was able to stratify the patients with high risk more precisely and provide further evidence for individual treatment.

The PPM members play different roles in cancer-related processes such as stress responses, apoptosis, and cell cycle [13]. Some of them are oncoproteins such as PPM1D (also known as Wip1) [14], while some are tumor suppressors such as PHLPP [15]. Moreover, one PPM member may have different functions in different tumor types because of the tissue and context-specificity. In our study, we showed that PPM1H was upregulated in NSCLC and resulted in a poor prognosis, suggesting that it participated in not only the oncogenesis but also tumor progression. However, the enrolled clinical variables such as tumor size or lymphatic invasion had no significant association with PPM1H expression, so how PPM1H influenced the oncogenic behavior of NSCLC should be further investigated to elucidate its role in NSCLC progression and prognosis.

As a new member of serine/threonine protein phosphatase, the substrates of PPM1H are not well elucidated and the molecular mechanism of PPM1H is almost unknown. Recent studies revealed that PPM1H was able to dephosphorylate Rab and tumor suppressor p27 [8, 16]. PPM1H dephosphorylated p27 at threonine 187 and decreased trastuzumab resistance in breast cancer [8]. Moreover, CSE1L, a proliferation and apoptosis-related protein, was a potential substrate of PPM1H [7]. Here we detected CSE1L and other well-accepted

progression-related biomarkers including p53 and Ki67, and analyzed the correlations between PPM1H. Interestingly, only Ki67 but not p53 and CSE1L, was significantly associated with PPM1H expression, suggesting that Ki67, may be involved in the processes that PPM1H promotes tumor progression. Ki67 is a well-known biomarker for cell proliferation, indicating that PPM1H may play an important role in the proliferation of NSCLC. However, there is no significant correlation between PPM1H and clinical factors such as tumor size or T stage (Table 2). Considering that tumor size and infiltration are affected by many variables, more *in vivo* and *in vitro* experiments should be performed to elucidate the molecular functions of PPM1H in NSCLC. In our study, we proved that PPM1H was a significant prognostic biomarker of NSCLC. The notable clinical significance of PPM1H in NSCLC would initiate more interest in the PPM1H function in NSCLC tumorigenesis and progression.

In conclusion, we showed that PPM1H expression was upregulated in NSCLC and identified it as an independent prognostic factor of NSCLC with a large cohort for the first time. Our result indicated that PPM1H was an important supplement of NSCLC molecular profile and detecting PPM1H may help recognize the high-risk patients for further treatment.

Supplementary information is available in the online version of the paper.

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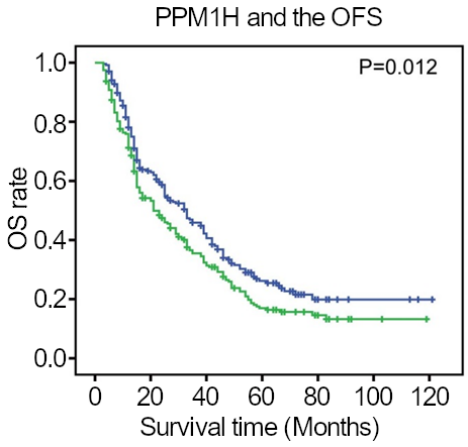
Supplementary Information



Supplementary Figure S1. The survival curves of PPM1H in NSCLC. The cohort was divided into low and high PPM1H expression according to the average level of IHC score. The accounts of patients with low and high PPM1H expression were 237 and 237, respectively.