Centromere protein F and Forkhead box M1 correlation with prognosis of non-small cell lung cancer

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Received May 16, 2019; Accepted October 15, 2019

DOI: 10.3892/ol.2019.11232

Abstract. Non-small cell lung cancer (NSCLC) is the most common histological type of lung cancer. Altered expression of centromere protein F (CENPF), a transient kinetochore protein, has been found in a variety of human cancers. However, its clinical significance in NSCLC remains unknown. In the present study the results of quantitative PCR and western blot analyses demonstrated that CENPF and Forkhead box M1 (FOXM1) were significantly higher in NSCLC tissues than in the non-cancerous controls at both transcriptional and translational levels. Immunohistochemical staining results showed 58.7% (44/75) and 64.0% (48/75) of NSCLC tissues displayed high expression of CENPF and FOXM1, respectively. CENPF protein expression showed a positive correlation with tumor size (P=0.0179), vital status (P=0.0008) and FOXM1 expression (P=0.0013) in NSCLC. Poor overall survival was correlated with high levels of CENPF and FOXM1 in NSCLC patients as evaluated by Kaplan-Meier and log rank test. Multivariate analyses showed that CENPF expression was an independent prognostic factor for NSCLC. In conclusion, our study provides evidence of the prognostic function of CENPF in NSCLC.

Introduction

Lung cancer is the second most common cancer and is the leading cause of cancer-related death for men and women worldwide (1-3). Non-small cell lung cancer (NSCLC), accounting for ~85% cases of lung cancer, represents the most common histological type of lung cancer (4). The mortality rate of NSCLC is very high, and the 5-year survival rate is <20% (5), which is due to the lack of reliable tools for early diagnosis or effective therapy. Therefore, investigation is required to identify specific molecules that may contribute to the diagnosis of NSCLC, and serve as prognostic markers.

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Key words: CENPF, FOXM1, prognosis, NSCLC

Centromere protein F (CENPF), a transient kinetochore protein, exhibits a cell-cycle dependent localization, and is completely degraded at the end of cell division (4-6). Evidence has shown that CENPF is overexpressed in head and neck squamous cell carcinomas, hepatocellular carcinoma, breast cancer and prostate cancer, and it may be a prognostic marker for these cancers (7-12). Forkhead box M1 (FOXM1) is a typical proliferation-associated factor and plays an important role in development (13-18). FOXM1 expression is frequently elevated in numerous malignancies and participates actively in the development and progression of various human cancers, including NSCLC (19-21). High expression of FOXM1 is correlated with shorter disease-free survival of NSCLC patients (22,23). The synergistic effect of FOXM1 and CENPF has been found in promoting the growth of prostate cancer and their co-expression predicts poor survival (24-26). However, the clinical significance of CENPF in NSCLC is unknown.

In the present study, the expression of CENPF and FOXM1 in NSCLC was explored by quantitative PCR, western blot analysis and immunohistochemical staining. The relationship between protein expression of CENPF and clinicopathological parameters were investigated to assess the possible prognostic value of CENPF and FOXM1 expression in NSCLC.

Patients and methods

Patients and clinicopathological data. The study was approved by the ethics committee of Shenyang Fifth People Hospital. A total of 75 patients with NSCLC who underwent surgery in Shenyang Fifth People's Hospital between 2009 and 2011 were enrolled after signed informed consent form was received. Tumor tissues and adjacent non-tumorous tissues were obtained from all the patients. Of these samples, 28 pairs of tumor tissues and adjacent non-tumorous tissues were frozen immediately, stored at -80˚C and used for quantitative PCR analysis. The samples were formalin-fixed, paraffin-embedded, and cut into 5-μm thick sections.

Quantitative PCR. Total RNA was isolated from collected tissues with TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Then, single-stranded cDNA was generated from 1 μg of total RNA using cDNA

synthesis kit (Thermo Fisher Scientific, Inc.). Quantitative PCR was carried out on ABI 7300 system (Applied Biosystem; Thermo Fisher Scientific, Inc.) with SYBR-Green qPCR Master Mixes (Thermo Fisher Scientific, Inc.) as per the manufacturer's instructions. The primers used in the study were: CENPF, forward, 5'-CTCGTTCCATCCCTGTCATC-3' and reverse primer 5'-TCCTGGTCAGATTCTCCTCC-3'; FOXM1, forward, 5'-GAAACGACCGAATCCAGAG-3' and reverse primer, 5'-GCAGATCGCCACTAAAGAAC-3'; GAPDH, forward, 5'-AATCCCATCACCATCTTC-3' and reverse primer 5'-AGGCTGTTGTCATACTTC-3'. CENPF and FOXM1 mRNA expression was calculated using the $2^{-\Delta\Delta Cq}$ method (27).

Western blot analysis. Total protein extracted from collected specimens (0.5 g per sample) was cut into small sections and homogenized in RIPA lysis buffer containing protease and phosphatase inhibitors (Beyotime). After centrifugation at 13,000 x g, at 4˚C for 20 min, the supernatant was recovered.

After separation by 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were electroblotted onto nitrocellulose membranes (Millipore) and subjected to western blot analysis, then incubated with primary antibodies, CENPF (rabbit polyclonal, 1:1,000 dilution, ab5), FOXM1 (rabbit polyclonal, 1:1,000dilution, ab226928) (both from Abcam) and GAPDH (rabbit monoclonal, 1:1,000dilution, no. 5174; Cell Signaling Technology), and membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibody (goat anti rabbit, 1:1,000 dilution, A0208; Beyotime). The immunoreactive signal was detected by the enhanced chemiluminescence kit (Millipore). Quantification of band intensity was analyzed with ImageJ software (http://rsb.info.nih.gov/ij/), and normalized to the intensity of GAPDH.

Immunohistochemical analysis. The sections were deparaffinized in xylene and hydrated in a graded series of ethanol, then antigen retrieved by heat exposure in Tris/EDTA buffer (pH 9.0) for 15 min, and blocked for endogenous peroxidase activity in 3% hydrogen peroxide at room temperature for 10 min. Followed by blocking with 5% normal blocking serum, the sections were reacted with anti-CENPF (1:200 dilution, ab5) (28) or anti-FOXM1 (1:250 dilution, ab207298) (both from Abcam) (29) at 4˚C overnight. After probing with HRP-conjugated secondary antibody, immunocomplexes were visualized using 3,3'-diaminobenzidine (DAB) (both from Long Island Biotech Co., Ltd.) and lightly counterstained with hematoxylin. The sections were reviewed and classified by two independent investigators as previously described (27). The percentage of positive stained cancer cells was scored as 0, negative; 1, 1-10%; 2, 11-50% positive; 3, >50% positive. The staining intensity was scored as 0, absent; 1, weak; 2, moderate; 3, strong. The immunoreactive score (IS) was calculated as follows: IS= percentage x staining intensity. The protein was considered to be highly expressed when IS was 3-9, otherwise to be lowly expressed.

Statistical analysis. Statistical analysis was carried out with the Statistical Package for the Social Sciences software (SPSS Inc.). Paired Student's t-test was performed to analyze the difference of mRNA and protein expression between NSCLC tissues and adjacent non-cancerous tissues. Pearson's correlation analysis was used to investigate the relationship between mRNA expression of CENPF and FOXM1 in NSCLC tissues. The Fisher's exact test was used to analyze the relationship between CENPF expression and the clinicopathological features. Kaplan-Meier and log rank test was used to estimate and compare survival. The significance level was set at 0.05.

Results

Association of CENPF and FOXM1 in NSCLC tissues. To describe the mRNA expression of CENPF and FOXM1 in NSCLC, we performed quantitative PCR analysis on 28 pairs of NSCLC tissues and adjacent non-cancerous tissues. Fig. 1A and B shows that 67.9% (19 cases) and 78.6% (22 cases) of patients showed high mRNA expression of CENPF and FOXM1, respectively. Paired Student's t-test revealed that mRNA levels of both genes were significantly elevated in NSCLC tissues compared to the non-cancerous tissues ($P<0.05$). Pearson's r correlation analysis displayed a significant positive association between CENPF and FOXM1 in NSCLC tissues ($P<0.0001$) (Fig. 1C).

mRNA profile data of lung cancer tissues and control tissues were downloaded from The Cancer Genome Atlas (TCGA) database. The expression of CENPF (Fig. 1D) and FOXM1 (Fig. 1E) was also significantly higher in lung cancer tissues than in normal control, and CENPF expression was positively correlated with FOXM1 expression in lung cancer tissues (Fig. 1F).

Furthermore, eight pairs of tissue samples were randomly selected from the above 28 pairs of samples and subjected to western blot analysis and the results confirmed the elevated protein levels of CENPF and FOXM1 in NSCLC tissues (Fig. 2A and B).

Elevated expression of CENPF correlated with clinical parameters of NSCLC. We further detected the protein expression of CENPF and FOXM1 in cancerous specimens and matched non-cancerous specimens from 75 NSCLC patients by immunohistochemistry. The clinical and pathological characteristics of these patients are listed in Table I. CENPF (Fig. 3A) and FOXM1 (Fig. 3B) expression was observed in cytoplasm and nucleus. Of the 75 patients, 58.7% (44 cases) and 41.3% (31 cases) showed high and low expression of CENPF, respectively, while 64.0% (48 cases) and 36.0% (27 cases) showed high and low expression of FOXM1, respectively.

The correlation between CENPF expression and the clinical parameters of NSCLC was analyzed by Fisher's exact test. As shown in Table II, CENPF protein expression was positively correlated with tumor size ($P=0.0179$), vital status ($P=0.0008$) and FOXM1 expression ($P=0.0013$), which suggested clinical significance of CENPF in NSCLC.

Expression of CENPF is closely related with the poor prognosis of patients with NSCLC. High expression of CENPF ($P<0.001$; Fig. 4A) and high expression of FOXM1 ($P<0.01$; Fig. 4B) in NSCLC were correlated with the short survival time of patients by Kaplan-Meier and log-rank test.

When both CENPF and FOXM1 were analyzed (Fig. 4C), patients whose tumors exhibited high expression of CENPF and high expression of FOXM1 had the shortest overall

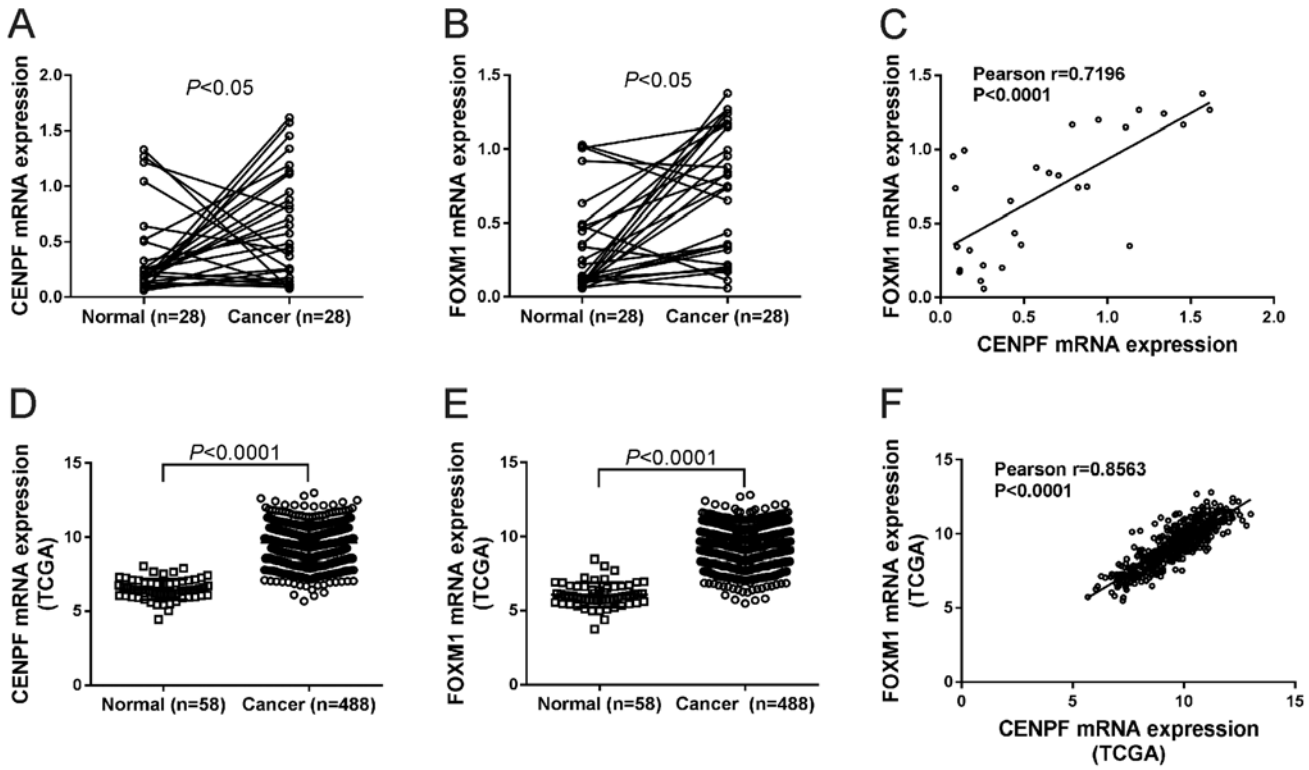

Figure 1. mRNA expression of CENPF and FOXM1 was elevated in NSCLC tissues. (A and B) mRNA expressions of CENPF (A) and FOXM1 (B) was analyzed by quantitative PCR on 28 pairs of NSCLC tissues and adjacent non-cancerous tissues. (C) Pearson's r correlation analysis between mRNA expression of CENPF and FOXM1 on 28 NSCLC tissues. (D-F) Analysis of CENPF (D) and FOXM1 (E) expression based on mRNA profile data from The Cancer Genome Atlas (TCGA) database. Pearson's r correlation analysis (F) between CENPF and FOXM1 was performed. NSCLC, non-small cell lung cancer; CENPF, centromere protein F; FOXM1, Forkhead box M1.

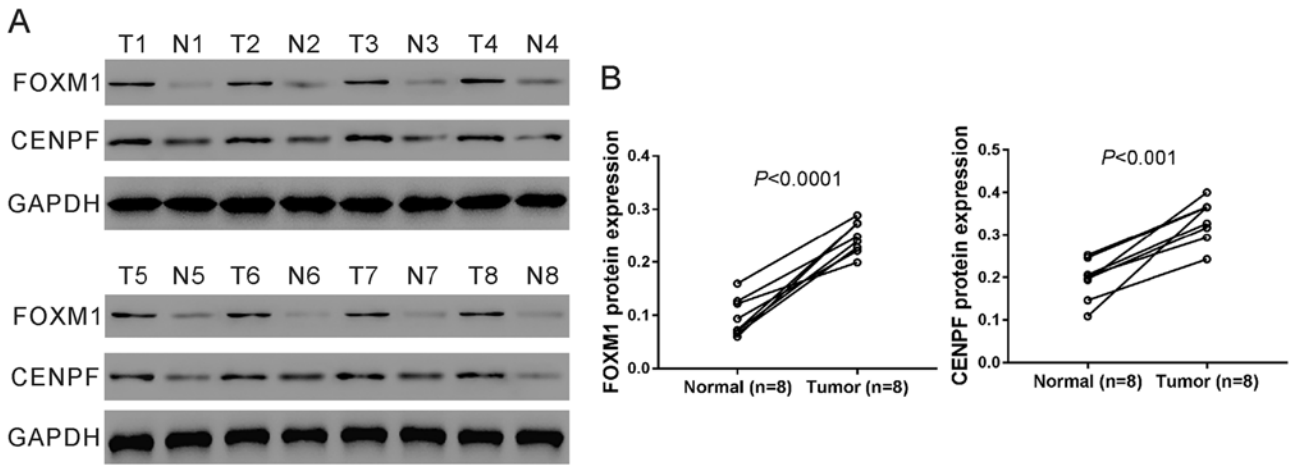

Figure 2. Protein expression of CENPF and FOXM1 was up-regulated in NSCLC tissues. Western blot analysis was performed to assess the protein levels of CENPF and FOXM1 on eight pairs of NSCLC (T1-T8) and non-cancerous tissues (N1-N8). GAPDH served as a loading control. Representative images (A) and quantitative analysis (B) of three independent experiments are shown. NSCLC, non-small cell lung cancer; CENPF, centromere protein F; FOXM1, Forkhead box M1.

survival time, whereas patients with tumors displaying low expression of CENPF and low expression of FOXM1 had the longest overall survival time (P<0.0001).

Finally, a multivariate Cox regression analysis was performed. CENPF (hazard ratio, 2.694; 95% CI, 1.397-5.195; P=0.003) was an independent parameter that was associated with overall survival when compared with tumor size and FOXM1 expression (Table III).

Discussion

Identification of specific biomarkers is important for diagnosis, therapy and prognosis of NSCLC. Previous studies have revealed the potential prognostic values of CENPF in several human cancers except NSCLC (7-12). In the present study, we pinpointed that CENPF expression was elevated in NSCLC tissues at both mRNA and protein levels. Then the

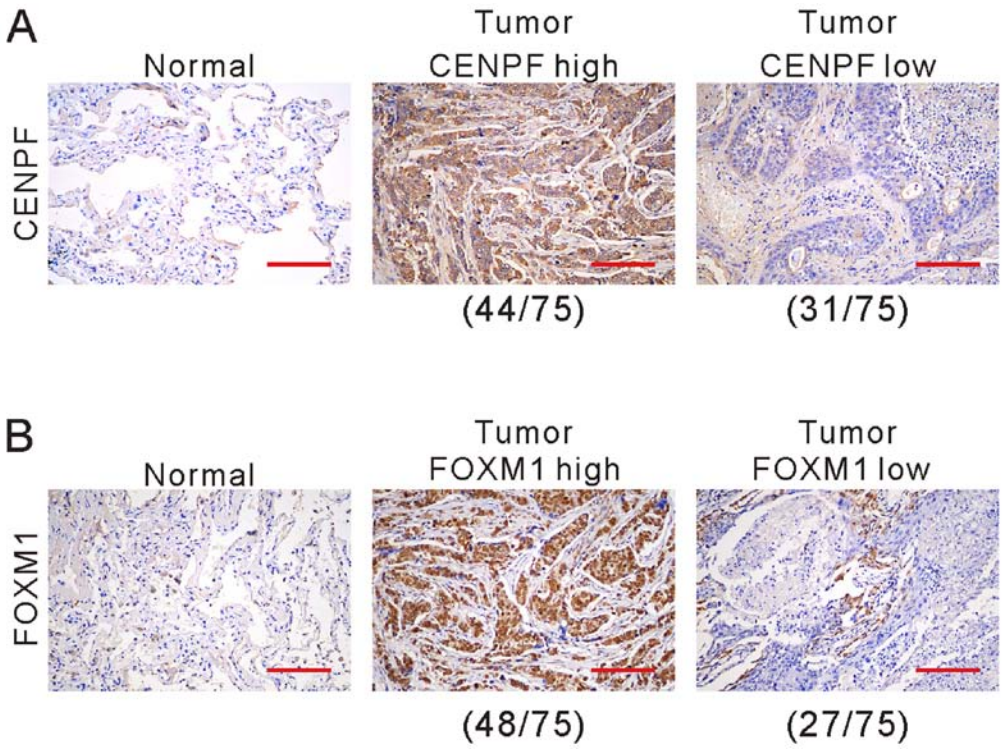

Figure 3. Immunohistochemical staining of CENPF (A) and FOXM1 (B) in NSCLC and non-cancerous tissues. The positive staining for CENPF and FOXM1 is brown in cytoplasm and nucleus. Nucleus is blue in hematoxylin counterstaining. Scale bars, 100 μm. NSCLC, non-small cell lung cancer; CENPF, centromere protein F; FOXM1, Forkhead box M1.

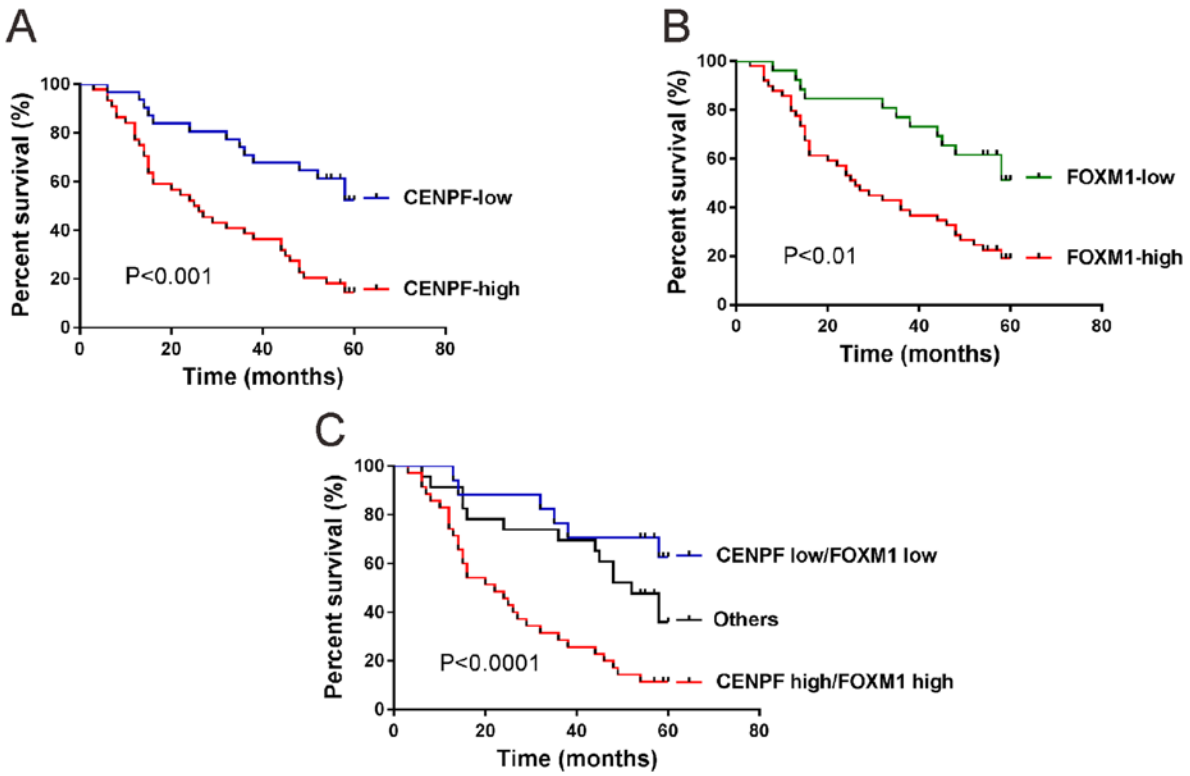

Figure 4. Kaplan-Meier survival plots in NSCLC patients (n=75). The patients were divided into different groups based on (A) CENPF expression, (B) FOXM1 expression, (C) CENPF and FOXM1 coexpression observed by immunohistochemical staining. NSCLC, non-small cell lung cancer; CENPF, centromere protein F; FOXM1, Forkhead box M1.

protein expression of CENPF in 75 cases of NSCLC and its association with overall survival and clinical characteristics were investigated. The results indicated that there was a significant correlation between CENPF expression and tumor

Table I. Clinicopathological characteristics in NSCLC patients (n=75).

Characteristic	Cases	%
Age (years)		
<60	37	49.3
≥60	38	50.7
Sex		
Male	42	56.0
Female	33	
Smoking status		
Smoker	25	33.3
Non-smoker	50	66.7
Tumor size		
<5 cm	31	41.3
≥5 cm	44	58.7
TNM stage		
I+II	30	40.0
III	45	60.0
Lymph node metastasis		
Absent	43	57.3
Present	32	42.7
Pathological type		
Adenocarcinoma	46	61.3
Squamous cell carcinoma	29	39.7
Vital status (at follow-up)		
Alive	24	32.0
Dead	51	68.0
CENPF expression		
Low	31	41.3
High	44	58.7
FOXM1 expression		
Low	27	36.0
High	48	64.0

NSCLC, non-small cell lung cancer; CENPF, centromere protein F; FOXM1, Forkhead box M1.

Table II. Correlation of CENPF expression in NSCLC tissues with different clinicopathological features (n=75).

	CENPF		
Characteristic	Low (n=31)	High (n=44)	P-value
Age (years)			0.6410
<60	14	23	
≥60	17	21	
Sex			0.8163
Male	18	24	
Female	13	20	
Smoking status			0.6210
Smoker	9	16	
Non-smoker	22	28	
Tumor size			0.0179[a]
<5 cm	18	13	
≥5 cm	13	31	
TNM stage			0.0991
I/II	16	14	
III	15	30	
Lymph node metastasis			0.3474
Absent	20	23	
Present	11	21	
Pathological Type			0.4705
Adenocarcinoma	21	25	
Squamous cell carcinoma	10	19	
Vital status (at follow-up)			0.0008[c]
Alive	17	7	
Dead	14	37	
FOXM1 expression			0.0013[b]
Low	18	9	
High	13	35	

Clinicopathological features were assessed using the Fisher's exact test. [a]P<0.05, [b]P<0.01, [c]P<0.0001. NSCLC, non-small cell lung cancer; CENPF, centromere protein F; FOXM1, Forkhead box M1.

size, vital status, and overall survival. Multivariate Cox regression analysis demonstrated that CENPF expression was an independent prognostic factor of patients with NSCLC. Our data suggest that CENPF expression may serve as a novel prognostic marker for NSCLC although further validation data with larger sample size are required.

FOXM1, a transcription factor, plays a critical role during development (13-18) and carcinogenesis (19-21). Previous studies have demonstrated that FOXM1 is an independent prognostic factor for NSCLC (22,23). FOXM1 and CENPF colocalized in the nucleus of prostate cancer cells, and co-expression of FOXM1 and CENPF is a prognostic indicator for poor survival of prostate cancer (24-26). In the present study, the findings also demonstrated the value of diagnosis and prognosis of FOXM1 in NSCLC. Importantly, we found that CENPF mRNA expression was

Table III. Multivariate Cox regression of prognostic parameters for survival in 75 NSCLC patients.

	Multivariate analysis		
Prognostic parameter	HR	95% CI	P-value
CENPF expression (low vs. high)	2.694	1.397-5.195	0.003[a]
Tumor size (<5 vs. ≥5 cm)	1.045	0.574-1.903	0.886
FOXM1 expression (low vs. high)	1.751	0.911-3.366	0.093

[a]P<0.01. HR, hazard ratio; CI, confidence interval; NSCLC, non-small cell lung cancer; CENPF, centromere protein F; FOXM1, Forkhead box M1.

positively correlated with FOXM1 mRNA expression in NSCLC samples by analyzing TCGA database and our own samples. CENPF protein expression was positively correlated with FOXM1 protein expression in NSCLC specimens as indicated by immunohistochemical staining. In addition, immunohistochemical staining analysis indicated a similar subcellular localization of CENPF and FOXM1 in NSCLC specimens. Patients with high expression of CENPF and FOXM1 had the worst overall survival, whereas patients with low expression of both proteins had the best overall survival. Thus, the present study suggests that CENPF and FOXM1 may co-operate in NSCLC. Aytes *et al* (24) reported that knockdown of CENPF decreased the binding of FOXM1 to its target genes as revealed by chromatin immunoprecipitation analysis. Similar mechanism may exist in NSCLC cells, which needs to be investigated in the future.

In conclusion, the present study has demonstrated that CENPF expression in NSCLC is correlated with FOXM1 expression and worse clinical outcome. These findings suggest that CENPF may function as a potential prognostic indicator for NSCLC. However, the present findings are based on a small sample size and further study with larger number of patients is needed.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

RL and YL wrote the manuscript. RL, XW and XZ performed PCR and western blot analysis. XZ and HC were responsible for immunohistochemical staining. YM and YL helped with statistical analysis. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Shenyang Fifth People's Hospital. Patients who participated in this research, signed an informed consent and had complete clinical data. Signed informed consents were obtained from the patients or the guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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