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.

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’-CTCCTTCCACCCTGTCATC-3’ and reverse primer 5’-TCCTGCGTACATTCCCTCC-3’; FOXM1, forward, 5’-GAACGGAGAAATCGAGAGG-3’ and reverse primer, 5’-GACGTGGGACTAAGAAGTGC-3’. GAPDH, forward, 5’-AATCCCATCACCTTTGTC-3’ and reverse primer 5’-AGGCTGTTGTCATACTTC-3’. CENPF and FOXM1 mRNA expression was calculated using the $2^{-\Delta\Delta C_{q}}$ 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, 1:1000 dilution, ab5), FOXM1 (rabbit polyclonal, 1:1000dilution, 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, 1:1000 dilution, A0208; Beyotime). The immunoreactive signal was detected by the enhanced chemiluminescence kit (Millipore). Photograph 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) 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, 1:1000 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.

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
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
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
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 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).

| 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           |            |             | 0.0179b |
| <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) |        |             | 0.0008c |
| Alive                | 17         | 7           |         |
| Dead                 | 14         | 37          |         |
| FOXM1 expression     |            |             | 0.0013b |
| Low                  | 18         | 9           |         |
| High                 | 13         | 35          |         |

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

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

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

*aP<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.

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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.

References
1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A: Global cancer statistics, 2012. CA Cancer J Clin 65: 87-108, 2015.
2. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J: Cancer statistics in China, 2015. CA Cancer J Clin 65: 115-132, 2016.
3. Zhang Y, Zheng T and Zhang W: Report of cancer incidence and mortality in China 4: 1-7, 2018.
4. Bjomont P, Maddox P, Shah JV, Desai AB and Cleveland DW: Unstable microtubule capture at kinetochores depleted of the centromere-associated protein CENP-F. EMBO J 24: 3927-3939, 2005.
5. Varis A, Salmela AL and Kallio MJ: Cenp-F (mitosin) is more than a mitotic marker. Chromosoma 115: 285-298, 2006.
6. Liao H, Winkfein RJ, Mack G, Rattner JB and Yen TJ: CENP-F is a protein of the nuclear matrix that assembles onto kinetochores at late G2 and is rapidly degraded after mitosis. J Cell Biol 130: 507-516, 1995.
7. Clark GM, Allred DC, Hilsenbeck SG, Chamness GC, Osborne CK, Jones D and Lee WH: Mitosin (a new proliferation marker) correlates with clinical outcome in node-negative breast cancer. Cancer Res 57: 5504-5508, 1997.
8. de la Guardia C, Casiano CA, Trinidad-Pinedo J and Báez A: CENP-F gene amplification and overexpression in head and neck squamous cell carcinomas. Head Neck 23: 104-112, 2001.
9. Kim HE, Kim DG, Lee JK, Son JG, Song MY, Park YM, Kim JJ, Cho SW, Chi SG, Cheong HS, et al: Frequent amplification of CENPF, GMNN and CDK13 genes in hepatocellular carcinomas. PLoS One 7: e43223, 2012.
10. O'Brien SL, Fagan A, Fox EJ, Millikan RC, Cullen HC, Brennan DJ, McCann AH, Hegarty S, Moyna S, Duffy MJ, et al: CENP-F expression is associated with poor prognosis and chromosomal instability in patients with primary breast cancer. Int J Cancer 120: 1434-1443, 2007.
11. Shahid M, Lee MY, Pipiani H, Andres AM, Zhou B, Yeon A, Kim M, Kim HL and Kim J: Centromere protein F (CENPF), a microtubule binding protein, modulates cancer metabolism by regulating pyruvate kinase M2 phosphorylation signaling. Cell Cycle 17: 2802-2816, 2018.
12. Li P, You S, Nguyen C, Wang Y, Kim J, Sirohi D, Ziembicke A, Luthringer D, Lin SC, Daskivich T, et al: Genes involved in prostate cancer progression determine MRI visibility. Theranostics 8: 1752-1765, 2018.
13. Wang IC, Snyder J, Zhang Y, Lander J, Nakafuku Y, Lin J, Chen G, Kalin TV, Whitsett JA and Kalinichenko VV: Foxm1 mediates cross talk between Kras/mitogen-activated protein kinase and canonical Wnt pathways during development of respiratory epithelium. Mol Cell Biol 32: 3833-3850, 2012.
14. Bolte C, Zhang Y, Wang IC, Kalin TV, Molkentin JD and Kalinichenko VV: Expression of Foxm1 transcription factor in cardiomyocytes is required for myocardial development. PLoS One 6: e22217, 2011.
15. Ustiiyan V, Wert SE, Ikegami M, Wang IC, Kalin TV, Whitsett JA and Kalinichenko VV: Foxm1 transcription factor is critical for proliferation and differentiation of Clara cells during development of conducting airways. Dev Biol 370: 198-212, 2012.
16. Kim I-M, Ramakrishna S, Gusarova GA, Yoder HM, Costa RH and Kalinichenko VV: The forkhead box m1 transcription factor is essential for embryonic development of pulmonary vasculature. J Biol Chem 280: 22278-22286, 2005.
17. Zeng J, Wang L, Li Q, Li W, Björkholm M, Jia J and Xu D: FoxM1 is up-regulated in gastric cancer and its inhibition leads to cellular senescence, partially dependent on p27 kip1. J Pathol 218: 419-427, 2009.
18. Yang C, Chen H, Tan G, Gao W, Cheng L, Jiang X, Yu L and Tan Y: FOXM1 promotes the epithelial to mesenchymal transition by stimulating the transcription of Slug in human breast cancer. Cancer Lett 340: 104-112, 2013.
19. Wang Z, Ahmad A, Li Y, Banerjee S, Kong D and Sarkar FH: Forkhead box M1 transcription factor: A novel target for cancer therapy. Cancer Treat Rev 36: 151-156, 2010.
20. Liu M, Dai B, Kang S-H, Ban K, Huang EJ, Lang FF, Aldape KD, Xie TX, Pelloski CE, Xie K, et al: FoxM1 is overexpressed in human glioblastomas and critically regulates the tumorigenicity of glioma cells. Cancer Res 66: 3593-3602, 2006.
21. Kim I-M, Ackerson T, Ramakrishna S, Tretiakova M, Wang IC, Kalin TV, Major ML, Gusarova GA, Yoder HM, Costa RH, et al: The Forkhead Box m1 transcription factor stimulates the proliferation of tumor cells during development of lung cancer. Cancer Res 66: 2153-2161, 2006.

22. Xu N, Jia D, Chen W, Wang H, Liu F, Ge H, Zhu X, Song Y, Zhang X, Zhang D, et al: FoxM1 is associated with poor prognosis of non-small cell lung cancer patients through promoting tumor metastasis. PLoS One 8: e59412, 2013.

23. Yang DK, Son CH, Lee SK, Choi PJ, Lee KE and Roh MS: Forkhead box M1 expression in pulmonary squamous cell carcinoma: Correlation with clinicopathologic features and its prognostic significance. Hum Pathol 40: 464-470, 2009.

24. Aytes A, Mitrofanova A, Lefebvre C, Alvarez MJ, Castille-Martin M, Zheng T, Eastham JA, Gopalan A, Pienta KJ, Shen MM, et al: Cross-species regulatory network analysis identifies a synergistic interaction between FOXM1 and CENPF that drives prostate cancer malignancy. Cancer Cell 25: 638-651, 2014.

25. Lin SC, Kao CY, Lee HJ, Creighton CJ, Ittmann MM, Tsai SJ, Tsai SY and Tsai MJ: Dysregulation of miRNAs-COUPTFII-FOXM1-CENPF axis contributes to the metastasis of prostate cancer. Nat Commun 7: 11418, 2016.

26. Lokody I: Signalling: FOXM1 and CENPF: co-pilots driving prostate cancer. Nat Rev Cancer 14: 450-451, 2014.

27. Lee JJ, Maeng CH, Baek SK, Kim GY, Yoo JH, Choi CW, Kim YH, Kwak YT, Kim DH, Lee YK, et al: The immunohistochemical overexpression of ribonucleotide reductase regulatory subunit M1 (RRMI) protein is a predictor of shorter survival to gemcitabine-based chemotherapy in advanced non-small cell lung cancer (NSCLC). Lung Cancer 70: 205-210, 2010.

28. Mizuno K, Matak i H, Arai T, Okato A, Kamikawaji K, Kumamoto T, Hiraki T, Hatanaka K, Inoue H and Seki N: The microRNA expression signature of small cell lung cancer: Tumor suppressors of miR-27a-5p and miR-34b-3p and their targeted oncogenes. J Hum Genet 62: 671-678, 2017.

29. Ma J, Qi G, Xu J, et al: Overexpression of forkhead box m1 and urokinase-type plasminogen activator in gastric cancer is associated with cancer progression and poor prognosis. Oncol Lett 14: 7288-7296, 2017.