Lamin B1 Overexpresses in Lung Adenocarcinoma and Promotes Proliferation in Lung Cancer Cells via AKT Pathway

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Purpose: This study aims to investigate the biological effect and molecular mechanism of Lamin B1 (LMNB1) in lung cancer cells and its significance for the prognosis of lung adenocarcinoma (LUAD) patients.

Methods: In this study, Bioinformatics was performed to analyze the expression at mRNA level and prognosis effect of LMNB1 in LUAD from TCGA dataset. The immunohistochemistry (IHC) assay was conducted to analyzed the expression of LMNB1 at the protein level in LUAD tissues. The correlation between the expression of LMNB1 and the clinical factors in patients with LUAD was analyzed. Next, LMNB1 transfected into LUAD cell lines (A549 and PC-9) which was proved by Western blot. CCK8 assay, cloning formation assay, and xenograft assay were conducted to explore the effect and mechanism of LMNB1 on the proliferation of LUAD cell lines in vitro and in vivo.

Results: The results of the present study demonstrated that LMNB1 was highly expressed in LUAD tissues and related to tumor stage. High LMNB1 expression was related with more advanced clinicopathological factors such as low degree of differentiation (P=0.02), large tumor size (P<0.01), lymph node metastasis (P<0.01) and higher tumor stage (P<0.01). After knocking down LMNB1, the cell growth rate (P<0.01) and the number of colonies (P<0.01) were significantly reduced, and the level of the proliferating marker Ki67 (P<0.01) was significantly decreased. At the same time, in vivo experiments showed that the tumor volume and tumor of the mice were significantly reduced (P<0.01). Moreover, we found that knockdown LMNB1 can inhibit the proliferation of lung cancer cells by inhibiting AKT phosphorylation by Western blot.

Conclusion: In summary, LMNB1 play an of vital roles in the growth of LUAD cells, highlighting its potential as a therapeutic target for the treatment of LUAD patients.

Keywords: lamin B1, lung adenocarcinoma, prognosis, malignant behavior

Introduction

According to the global cancer statistics for 2018, lung cancer is still the most frequently diagnosed cancer and the major cause of cancer death, accounting for 2.1 million new lung cancer cases and 1.8 million deaths in 2018.1 In various pathological types of lung cancer, lung adenocarcinoma is the primary pathological type causing 500,000 deaths worldwide each year.2 With the continuous development of high-throughput sequencing, biomolecular-based diagnosis and targeted therapy for lung cancer have also achieved remarkable results. However, due to the heterogeneity of tumors and drug resistance, the diagnosis of early lung cancer and
the treatment of advanced lung cancer are far from ideal, the five-year survival rate of lung adenocarcinoma is only 15%. More and more studies have demonstrated that the tumorigeness and development of lung cancer involve various signaling pathways. Therefore, it is necessary to find key molecules to guide clinicians to make accurate diagnosis and treatment.

As the evolutionary progenitors of the intermediate filaments supergene family, the lamins are classified as type IV intermediate filament proteins. Lamins, which are divided into A and B types, are located in the nuclear membrane and their main function is to stabilize the binding of protein and chromatin. Recently, more and more studies have indicated that Lamin B1 are abnormally expressed in multiple types of tumors such as hepatocellular carcinoma, colon cancer, cervical cancer and breast cancer. These studies indicate that Lamin B1 can be used clinically as a novel tumor biomarker to guide physicians in developing treatment strategies and accurately predicting patient outcomes. Yang et al identified a novel miRNA named MIR-G-1 which can target and up-regulating Lamin B1. This process promoted autophagy in the nucleus induced by serum starvation. It indicated that overexpression of Lamin B1 might play an of vital role in malignant behavior of cervical cancer cells. Except that, the AKT pathway plays a key role in the regulation of multiple cellular life activities such as proliferation survival, invasion and differentiation. Many tumors including lung cancer promotes tumorigenesis and inhibits apoptotic signals by activating AKT pathway. And genetic alterations of AKT is a common phenomenon in LUAD, and the mutation contains AKT1, AKT2 and AKT3. The PI3K/AKT pathway also participate in a series of cellular process such as cell metabolism, cell senescence, cell survival and angiogenesis. Therefore, the alterations of PI3K/AKT pathway and its downstream effectors are important reasons promoting tumor growth and metastasis. AKT phosphorylates which was involved in cell survival, also leads to cell proliferation ability alterations. This process was caused by AKT induced pro-apoptotic proteins downregulations. But the role of Lamin B1 in AKT pathway of LUAD has not been elucidated, and until now, there is no report on Lamin B1 molecular mechanism in the oncogenesis process of LUAD and its effect on the prognosis of LUAD patients.

In this study, we analyzed the expression level of Lamin B1 in LUAD tissue and its effects on the prognosis of LUAD patients from TCGA database and clinical information of patients undergoing radical resection of lung cancer in our hospital, and analyzed the mechanism of Lamin B1 in the development of LUAD and the relationship between Lamin B1 and AKT pathway in vitro and in vivo using lung adenocarcinoma cell lines, respectively.

Materials and Methods

Bioinformatics Analysis

The data of differential analysis of LMNB1 expression at mRNA level was obtained from the TCGA database including 483 LUAD tissues and 347 normal lung tissues. GEPIA (gepia.cancer-pku.cn) was performed to generates box plots with jitter for comparing the expression of LMNB1 between LUAD tissues and normal lung tissues. Firstly, the expression data were log2(TPM+1) transformed for differential analysis and the log2FC is defined as median (Tumor)/median (Normal) (TPM: Transcripts Per Million, FC: fold changes). Genes with higher log2FC values than pre-set thresholds (fold changes is 2 times) were considered differentially expressed genes. We also generate expression violin plots based on patient pathological stage. The method for differential gene expression analysis is one-way ANOVA, using the pathological stage as the variable for calculating differential expression. A value of Prob (>F) of less than 0.05 indicates a statistical difference. The survival analysis was also obtained from the TCGA database by using the Kaplan-Meier method. The patients were divided into the high group (n=120, in TCGA database) and low group (n=120, in TCGA database) according to the quartile Lamin B1 expression at mRNA level as the cutoff point.

Clinical Specimen Collection

Lung adenocarcinoma tissue specimens and paracancerous lung tissue specimens were collected from 86 LUAD patients who underwent radical resection of lung cancer at Tianjin First Center Hospital from 2017 to 2018. Patients who received preoperative radiotherapy or chemotherapy were excluded. All clinical data of 86 LUAD patients are available. This study was approved by Tianjin First Central Hospital Medical Ethics Committee with strict adherence to the principles of the Declaration of Helsinki. The informed consent will inform the patients and their immediate family members. All patients and their immediate family members have signed the informed consent.
Cell Culture and in vitro Transfection

Human LUAD cell lines (PC-9, A-549, and H1975) and human bronchial epithelial cells line, BEAS-2B, were purchased from American Type Culture Collection. PC-9, A-549, and H1975 were cultured in Roswell Park Memorial Institute 1640 (BI, 01-100-1A, Israel) with 10% fetal bovine serum (BI, 04-100-1A, Israel) and BEAS-2B was cultured in high glucose Dulbecco’s modified Eagle’s medium (BI, 01-056-1A, Israel) with 10% fetal bovine serum (BI, 04-100-1A, Israel). For knocking down LMNB1, lentiviral-based vector (V2LHS_62675, 5'-GCATTAAGCAGCGTATC-3', Lafayette, USA) was used. 48 hrs after virus transfection, stably transfected cell strains were screened with puromycin (2 μg/mL), and the transfection efficiency was verified by Western blot assay.

RNA Extraction and Quantitative Real-Time PCR

Trizol reagent (Invitrogen, 15596026, USA) was conducted to separate total RNA from fresh tissues. High-Capacity cDNA Reverse Transcription (ThermoFisher, 4368813, USA) was used to synthesize complementary DNA (First-Strand) according to the manufacturer’s protocol. Quantitative real-time PCR assay was conducted to analyze the amount of cDNA by using KAPA SYBR FAST kit (Merck, KK4601, Germany). Primers were: LMNB1 Forward: 5'-TCGCAAAAGCATGTATGAAGA-3'; Reverse: 5'-CTCTACCAAGCGCAGCATGTATGAAGA-3'; and GAPDH Forward: 5'-AGCCACATCGCTGAGCATGTATGAAGA-3'; and GAPDH Forward: 5'-GCCAAATACGACCAAACTCTACCAAGCGCAGCATGTATGAAGA-3'; Reverse: 5'-GCCAAATACGACCAAACTCTACCAAGCGCAGCATGTATGAAGA-3'. The 2^{-ΔΔCT} method was used to calculate the expression level of LMNB1, and GAPDH was used as an internal control.

Immunohistochemical Staining

Fresh tumor tissue was immersed in formalin and then embedded in paraffin. The thickness of tissue sections was 4 μm, and routine dewaxing, hydration and antigen repair were performed. Catalase was blocked by 3% hydrogen peroxide. And the sections were incubated with LMNB1 antibodies (1:300, Abcam, ab133741, UK) overnight at 4°C. The next day, sections were incubated with secondary antibody (ZSGB-BIO, PV-6000, China) after DAB kit (Solarbio, DA1010, China) staining. IHC staining was scored using a semi-quantitative method according to the previous study.17

Western Blot

RIPA buffer was conducted to lyse the harvested cells which were washed with PBS. BCA kit was used to determine the protein concentration of each sample. 30 μg proteins were separated on 8% sodium dodecyl sulfate-polyacrylamide gel and transferred to poly (vinylidene fluoride) membranes at 4 °C. TBST was used to wash the membrane for 3 times at room temperature, and primary antibodies were performed to incubate with the membrane at 4 °C overnight. The primary antibodies as follows: LMNB1 (1:5000, Abcam, ab133741, UK), Ki67(1:2000, Abcam, ab16667, UK), pAKT (1:2000, ABclonal, AP0140, USA). Then the secondary antibodies were performed to incubate with membranes at room temperature for 1h. The ECL mixture was used to develop the blots and Imager was used to visualize the blots.

Cell Proliferation Assay

Cell counting kit-8 (Bimake, B34302, China) was conducted to determine the cell proliferation ability of cell lines. Briefly, the cells in the logarithmic growth phase were seeded in 96-well plates at 1000 per well. After 6 days of culture, 10 μL of the cell counting kit-8 reagent was added to each well. Continue to culture for 1–4 hrs until the color turns orange. The 450 nm light absorption value was read with a microplate reader. Repeat three experiments in each group.

Cloning Formation Assay

The cells were collected in the logarithmic growth phase of the cells and seeded in a six-well plate at 900 cells per well. After one week of culture, fixation was carried out using 4% paraformaldehyde, and the cells were stained with crystal violet (Solarbio, C8470, China).

Xenograft Mice Model in vivo

6–8 weeks old BALB/C nude mice were purchased from HFK Bioscience company (Beijing, China). The shCon and shLMNB1 A-549 cells which were re-suspended with 100 μL PBS (5x10^6 cells/mice) were subcutaneously injected into the left inguinal region of mice, each group had 3 mice. About 1 week later, tumors can be touched. Then we measure the size of tumors with Vernier calipers every three days. About five weeks later, the mice were executed and the tumor was taken out for Western blots. The formula V=1/2a x b^2 was used for calculating the volume of the tumor. All the animals experiment was approved by Tianjin
First Central Hospital Animal care and use committee. In order to reduce the suffering of laboratory animals, all efforts were made according to the Guidelines for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006).

**Statistical Analysis**

Statistical analysis was conducted using SPSS 22.0 software (IBM SPSS, Chicago, USA). The chi-square test was used to analyze the relationship between LMNB1 expression at the protein level and clinical pathologic features of LUAD patients. The differences of data between groups were performed by using Student’s t-test and one-way ANOVA test (Data follow a normal distribution). The data which do not follow normal distribution from different groups were compared by using the Mann–Whitney U-test and Kruskal–Wallis test. Differential analysis of LMNB1 expression at mRNA level and survival analysis was performed by using GEPIA website (http://gepia.cancer-pku.cn). The independent prognostic factors for OS and PFS was determined by using the cox proportional hazard model. Two sides P values ≤ 0.05 was considered significant.

**Results**

**LMNB1 Expression Is Up-Regulated in LUAD**

First, we compared the expression of LMNB1 at mRNA level between tumor tissues and normal tissues from the TCGA database by using bioinformatics methods. As shown in Figure 1A, LMNB1 expression in LUAD tissues was twice times higher than normal tissues (The expression of LMNB1 in one group is more than twice that in the other group, and the figure will show red star). Except that, bioinformatics analysis showed that the expression level of LMNB1 increased with the clinical stage of LUAD patients as shown in Figure 1B (Pr<0.05 indicates a statistical difference). To verify the results of bioinformatics analysis, we detected the expression of LMNB1 in cell lines and tissues, respectively. As shown in the Figure 1C, the expression level of LMNB1 in normal pulmonary epithelial cell line BEAS-2B was obviously lower than that in other three LUAD cell lines, the expression level of LMNB1 on the A549 and PC-9 cell line is high. Then, qPCR was conducted to measure the expression of LMNB1 at mRNA level in 14 fresh tumors and 15 fresh adjacent tumor tissues. The results demonstrated that the expression of LMNB1 in tumors increased significantly (Figure 1D). Meanwhile, we detected the difference of LMNB1 protein expression in 86 LUAD tissues and 14 adjacent tissues by IHC assay. The data also demonstrated that the expression of LMNB1 in LUAD tissues increased significantly (Figure 1F and E). Both the results of the database and our experiments prove that LMNB1 is highly expressed in LUAD tissues.

**Knockdown of LMNB1 Can Suppress the Proliferation of LUAD Cells via AKT/ pAKT Pathway in vitro**

In order to further study the biological effect of LMNB1 on LUAD cells, we used shRNA to knock down LMNB1 in A549 and PC-9 cell lines. As shown in Figure 2A, the knockdown efficiency of LMNB1 was validated by Western blot at the protein level. Then CCK8 assay and colony formation assay was performed to explore the effect of knocking down LMNB1 on the proliferation of A-549 and PC-9 cell lines, and Figure 2B and C revealed that the cell proliferation level and colony number in the shLMNB1 group were significantly lower than those in the shCON group. Interestingly, Western blots assay revealed that the levels of Ki67 and pAKT in shLMNB1 group decreased significantly, but the levels of total AKT did not change (Figure 2D and E). This result further confirmed that knocking down LMNB1 could suppress the proliferation in both A549 and PC-9 cells, and the pAKT/ AKT pathway played a vital role in this proceeding.

**Knocking Down LMNB1 Can Suppress Tumor Growth in vivo**

In vitro experiments have confirmed that LMNB1 plays a vital role in the proliferation of A549 cells. However, the role of LMNB1 in oncogenesis and development in vivo remained elusive. Hence, we established xenograft tumor-bearing mice model by injecting shCON A-549 cells and shLMNB1 A-549 cells subcutaneously. As shown in Figure 3, the mice were euthanized five weeks later, and the volume and weight of the tumors were measured. As shown in Figure 3A–C, tumor weight and growth rate of shLMNB1 group were remarkably reduced compared to shCON group. What’s more, Western blot revealed that the protein level of Ki67 and pAKT in tumor tissues were significantly down-regulated after knocking down LMNB1. Similar to in vitro results, the level of total AKT protein did not change significantly after LMNB1 knockdown (Figure 3D and E). The IHC also demonstrated that Ki67 were decreased after knocking down LMNB1 (Figure 3F). This part of the data demonstrated that LMNB1
Figure 1. LMNB1 was upregulated in LUAD tissues compared with paracancerous tissues (A) The expression of LMNB1 at mRNA level in LUAD patients from TCGA dataset by using bioinformatics’ analyses (* means differentially expressed genes). (B) The expression of LMNB1 in the different pathological stage of LUAD patients from TCGA dataset by using bioinformatics’ analyses. (C) Expression of LMNB1 in LUAD cell lines and human bronchial epithelial cells line by using Western blot. The histogram is a semi-quantitative analysis of Western blot data (**P<0.01). (D) The expression of LMNB1 at mRNA level in LUAD patients by qPCR. (E) Representative IHC staining images of LMNB1 in LUAD tissues and normal lung tissues. (F) Chart of expression of LMNB1 by using IHC and the associated statistics (***P<0.001).
could regulate the proliferative capacity of tumor cells via pAKT/AKT pathway, and thus play a key role in the development of LUAD. LMNB1 may be served as a potential therapeutic target for LUAD patients.

High Expression of LMNB1 Is Associated with Worse Clinicopathological Features in LUAD Patients

As shown in Table 1, the relationship between LMNB1 expression and clinicopathological features in LUAD patients are presented. Interestingly, the data showed that LMNB1 overexpression was associated with low degree of differentiation (P=0.02), large tumor size (P<0.01), lymph node metastasis (P<0.01) and higher tumor stage (P<0.01). However, the relationship between LMNB1 overexpression and age (P=0.96), gender (P=0.99), and smoking history (P=0.39) was not statistically significant.

Increased LMNB1 Expression Is Related to the Adverse Overall Survival of LUAD Patients

To confirmed whether the expression at the mRNA level of LMNB1 is associated with disease-free survival (DFS) and overall survival (OS) in LUAD patients, we used bioinformatics methods to analyze the survival of LUAD patients from the TCGA dataset. We used the quartile LMNB1 mRNA expression level as the cutoff value and divided the patients into the LMNB1 high expression group (n=120) and the LMNB1 low expression group (n=120). Then, Kaplan-Meier's method was used to compare the OS and DFS of the two groups. As shown in Figure 4, there was an obvious difference in OS between the two groups (P=0.022, Figure 4A); however, there was no statistically significant difference in disease-free survival between the two groups (P=0.099, Figure 4B). To further explore LMNB1’s prognostic value in LUAD, univariate and multivariate cox proportional hazards regression were performed to analyze the clinical data of LUAD patients from the TCGA database. As shown in Table 2, we found that LMNB1 (P=0.005), pT-stage (P<0.001), and clinical stage (P<0.001) were identified as a prognostic factor for overall survival by univariate analysis. Multivariate analysis also identified LMNB1 (P=0.005), pT-stage (P=0.01), and clinical stage (P<0.001) as an independent prognostic factor for OS in patients with LUAD.
Lung cancer ranks first among cancer-related deaths in developed countries, and about 25% of cancer deaths are caused by lung cancer according to the 2019 cancer statistics. Lung adenocarcinoma (LUAD), which belongs to non-small-cell lung cancer (NSCLC), is the main pathological subtype of lung cancer. Among them, LUAD accounts for 40% of the total number of newly diagnosed lung cancer. In recent

**Discussion**

Lung cancer ranks first among cancer-related deaths in developed countries, and about 25% of cancer deaths are caused by lung cancer according to the 2019 cancer statistics. Lung adenocarcinoma (LUAD), which belongs to non-small-cell lung cancer (NSCLC), is the main pathological subtype of lung cancer. Among them, LUAD accounts for 40% of the total number of newly diagnosed lung cancer. In recent
years, for advanced and recurrent lung adenocarcinoma, molecular targeted therapy and immunotherapy have improved the clinical prognosis of patients to some extent. Yet, the effect of oncogene on the tumorigenesis of lung adenocarcinoma and resistance mechanism are still not be fully elucidated. At the same time, patients and society bear high medical and health costs. Therefore, it is urgent to find effective oncogenes to guide the respiratory doctor to make accurate diagnosis and treatment for different patients.

More and more studies have confirmed the abnormal expression of LMNB1 in colorectal cancer, hepatocellular carcinoma, breast cancer, lymphoblastic leukemia, and pancreatic cancer. And many studies have demonstrated that LMNB1 play a vital role in carcinogenesis. However, there is no research on the association between LMNB1 and LUAD until now. In this study, we aimed to investigate the effect of LMNB1 in the progress of LUAD. Firstly, the data revealed that LMNB1 was abnormally high expressed in LUAD tissues and was related with the clinical stage of LUAD by bioinformatics. Then, we validated the above conclusions by using Western blot assay, qPCR assay, and IHC assay, and found that LMNB1 was abnormally high expressed in LUAD cell lines and tissues. In order to further analyze the role of LMNB1 in the progress of LUAD, we demonstrated that LMNB1 could regulate the proliferation of LUAD cells via AKT pathway, respectively. At last, we found that overexpression of LMNB1 predicted adverse overall survival in LUAD patients by using K-M methods.

LMNB1 is a member of lamin protein family. It is an architectural protein which is involved in chromatin regulation, protein interactions, cytoskeletal connections, DNA damage and repair. But its effect on tumorigenesis and tumor development is still unclear. Due to the misregulation of LMNB1 in many cancers, LMNB1 has become popular in tumor biomarker research. In Sun’s study, they found that LMNB1 was significantly elevated in tumors and plasma of patients with hepatocellular carcinoma (HCC) by proteomic-wide profiling and was associated with tumor sizes, tumor stages, and tumor nodules. Except that, they demonstrated that early stage HCC can be found by detecting the expression level of LMNB1 from the plasma. In our study, we also found that LMNB1 was upregulated in the LUAD tissues and was associated with adverse clinical pathological features such as low degree of differentiation, large tumor size, and lymph node metastasis in patients with LUAD. And overexpression of LMNB1 was associated with adverse overall survival in LUAD patients. Li et al demonstrated that knocking down LMNB1 could attenuate the proliferation and metastasis abilities of pancreatic cancer cells and betulinic acid therapy could downregulate the expression of LMNB1 which is independent of transcription factor SP1. As is known to all, the PI3K/AKT/mTOR pathway which is activated by the binding of EGF (extracellular growth factors) to RTKs (transmembrane receptor tyrosine kinases) play a crucial role in regulating cell proliferation, adhesion, metastasis, differentiation, and metabolism. Except that, the abnormal regulation of this pathway is related to the tumorigenesis of lung cancer, high-grade tumor and advanced progression. Interestingly, our data also demonstrated that LMNB1 could modulate the proliferation ability of LUAD cells via AKT pathway both in vitro and in vivo. Therefore, we believed that LMNB1

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Note: *Statistical significant.
might play a crucial role in the proceeding of carcinogenesis as an oncogene. Liu’s study screened out seven tumor-associated protein including LMNB1 by using T7 phage display system and found that combined detection of these autoantibodies was an auspicious way for finding early hepatocellular carcinoma. Except that, Michalak et al used proteomics to analyze changes in different proteins from colon cancer cell lines treated with galectin-4 and found that the level of LMNB1 was significantly down-regulated. Hence, LMNB1 plays a facilitating role in the growth of colon tumors. Andersen et al identified three risk genes associated with childhood leukemia recurrence from pre-selected potential clinically relevant single-nucleotide polymorphisms, including LMNB1. But in Izdebska et al study, they considered that LMNB1 upregulation would result in cell death via mitotic catastrophe pathway and inhibition of cell migration in colon cancer. And Wazir et al also demonstrated that knocking down LMNB1 was related with adverse DFS in breast cancer. Therefore, the role of LMNB1 in the process of carcinogenesis from various types of cancer is quite different.

In conclusion, we have demonstrated that LMNB1 is highly expressed in LUAD tissues when compared with paracancerous tissues, which can modulate the proliferation ability on LUAD cells and predict adverse prognosis. Therefore, we believed that the high expression of LMNB1 may affect the size and stage of tumors in LUAD patients, thereby affecting its clinical prognosis. LMNB1 might become a potential therapeutic target for LUAD. Yet, there is also obvious deficiency: The detailed mechanism of how LMNB1 regulates tumor proliferation needs further explanation. Due to the follow-up time of

![Figure 4](https://www.dovepress.com/)

Figure 4 Upregulated LMNB1 indicates an adverse prognosis in LUAD patients. (A) Overall survival analysis of LUAD patients from TCGA dataset (P=0.022). (B) Disease-free survival analysis of LUAD patients TCGA dataset (P=0.099).

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**Notes:** *P value was analyzed by Chi-square test; *Indicates P<0.05 with statistical significance.
LUAD patients in our institution is too short, we need to conduct large sample and long-term follow-up to further verify our conclusion.

Data Sharing Statement
The data used to support the findings of this study are available from the corresponding authors upon request.

Disclosure
The authors report no conflicts of interest in this work.

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