ZNF488 Enhances the Invasion and Metastasis through Activating the MAPK/ERK Signaling Pathway in Non-Small Cell Lung Cancer

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Abstract

**Background:** This study aimed to explore the relationship between zinc finger protein 488 (ZNF488) and non-small cell lung cancer (NSCLC), as well as the function and mechanism of ZNF488 involved in the invasion and metastasis.

**Method:** TCGA and HPA databases were used to analyze NSCLC data. Quantitative Reverse transcription polymerase chain reaction (qRT-PCR) was used to detect the expression level of ZNF488 in tissues. 100 cases of NSCLC tissues were subjected to immunohistochemical staining (IHC) to evaluate the endogenous expression of ZNF488, and then analyze its correlation with clinical characteristics. After constructing ZNF488 stably overexpressing cell lines, wound healing test and transwell invasion assays were used to explore the invasion and migration ability of lung cancer cells in both ZNF488 over-expressing and the vector control cell lines. In vivo, we used ZNF488 over-expressing and the vector control cell lines to construct animal models of lung metastasis by tail vein injection. The gene chip technology was used to explore the differential genes between vector and ZNF488 over-expressing group. The DAVID database was used to analyze the differential genes by KEGG pathway MEK inhibitor AZD6244 was used to verify the potential signaling pathway. Western blot was used to detect the protein expression levels.

**Results:** The expression of ZNF488 in NSCLC tissues was generally higher than that in normal lung tissues, and its expression level was significantly related to TNM stage, overall survival (OS), local recurrence-free survival (LRFS), distant metastasis-free survival (DMFS), and progression-free survival (PFS) ($p<0.05$). The results of functional and animal experiments showed that ZNF488 promoted the cell invasion and metastasis in NSCLC. In terms of mechanism, ZNF488 can induce epithelial-mesenchymal transition (EMT) in NSCLC by activating the MAPK/ERK signaling pathway, thereby promoting the invasion and metastasis. MEK inhibitor AZD6244 could partially reverse ZNF488-induced invasive ability.

**Conclusion:** As an independent prognostic indicator, ZNF488 can promote the invasion and metastasis by activating the MAPK/ERK signaling pathway in NSCLC.

**Background**

Lung cancer is the malignant tumor with the highest mortality rate and the second morbidity rate in the world. The incidence has gradually become younger, which is a serious threat to human health$^{[1-2]}$. Although some progress has been made in the diagnosis and treatment, recurrence, but distant metastasis are still the main causes of death$^{[3-7]}$. Therefore, finding relevant tumor molecular markers for predicting the progression of lung cancer is a research hotspot in recent years$^{[8-10]}$.

EMT plays an important role in the process of tumor invasion and metastasis. When tumor cells undergo EMT, the cell polarity is weakened and the adhesion ability decreases, so that the corresponding mesenchymal cell characteristics are obtained meanwhile the invasion and migration ability is
A large number of studies have confirmed that the EMT process of lung cancer cells is an important reason for their invasion and metastasis\textsuperscript{\cite{12-15}}. It has been confirmed that TGF-β, PI3K/AKT, RAS-MAPK and other signaling pathways are involved in this process\textsuperscript{\cite{16-18}}. When the signal pathway is activated, the expression levels of epithelial markers E-cadherin and α-Catenin of tumor cells decrease, while the expression levels of mesenchymal markers vimentin and N-cadherin increase\textsuperscript{\cite{19-20}}. These changes lead to the weakening of tight junctions between cells and the enhancement of invasion and migration capabilities\textsuperscript{\cite{20}}.

As a transcription factor, zinc finger proteins can specifically bind to target genes, themselves or other zinc finger proteins through finger-like domains. Then, they participate in the regulation of gene expression, cell differentiation and other biological functions\textsuperscript{\cite{22}}. ZNF488 is located in the q11.22 segment of human chromosome 10, with a total length of 3513 bp. The encoded gene consists of 340 amino acids and has a molecular weight of 38 kDa\textsuperscript{\cite{23-24}}. Studies have shown that ZNF488 can act as an oncogene to activate the Wnt pathway and induce EMT in nasopharyngeal carcinoma cells, thereby promoting the tumor progression of nasopharyngeal carcinoma\textsuperscript{\cite{25}}. However, the role and mechanism of ZNF488 in the progression of lung cancer are still unclear. This study aimed to explore the relationship between ZNF488 and NSCLC, as well as the function and mechanism of ZNF488 involved in the invasion and metastasis in NSCLC.

**Material And Methods**

**1. Cell culture**

All NSCLC cell lines were obtained from the Central Laboratory of Jiangsu Cancer Institute (Nanjing, Jiangsu). These cells were grown in DMEM/F12 or RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and streptomycin. All cell lines were cultured at 37 °C, 5% CO\textsubscript{2} in humid air, and conducted a mycoplasma contamination test every 3 months. The cells used in the experiment were all within 10 generations after thawing.

**2. Tissue samples**

The 100 NSCLC tissue samples included in the study were provided by Jiangsu Cancer Hospital and confirmed by experienced pathologists. According to the Declaration of Helsinki, this study had been approved by the Clinical Ethics Review Committee of Jiangsu Cancer Hospital, and a written informed consent form of each patient involved in the study was obtained. The histological grade and staging of NSCLC were reclassified according to the 8th edition of the American Joint Committee on Cancer (AJCC) cancer staging system.

**3. Generation of plasmids and stably transfected cell lines**
NCBI database was used to analyze the full length of ZNF488 gene sequence. Primer bank website was used to design ZNF488 primers: Forward: 5'-GAAAACAGATGGCGACTTAGCG -3'; Reverse: 5'-CTGCCGGTCTTCTCCTCCTC-3'. ZNF488 cDNA was amplified by PCR and insert it into pPMCV vector. The vector or pPMCV-ZNF488 plasmid was transfected together with the retroviral packaging vector PIK into 293FT cells. After transfection, the supernatant was collected and used to infect NCI-H520 and PC-9 cells, and the stable transfected cell lines were screened with puromycin.

4. RNA extraction, reverse transcription and qRT-PCR

Trizol reagent (Invitrogen) was used to isolate total RNA from tissues and cells. Reverse transcription was performed using M-MLV reverse transcription mold (Promega, Madison, WI). Quantitative RT-PCR was performed using the Bio-RAD CFX96 real-time system (Bio-Rad, Hercules, CA) and Platinum SYBR Green Qpcr SuperMix-UDG reagent (Invitrogen). Then extract RNA according to the instructions provided by the reagent supplier.

5. Western blot analysis

The total protein was separated on a 6%-12% SDS-polyacrylamide electrophoresis gel and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). Block the membrane with 5% skimmed milk and incubate with the primary antibody against ZNF488 (Abcam), then incubate with the anti-mouse or anti-rabbit IgG secondary antibody. The band is detected by enhanced chemiluminescence, and GAPDH or α-tubulin is served as a loading control.

6. Wound healing assay

When the cells in the 6-well plate grow to 80-90% confluence, replace the complete medium with serum-free medium, and incubate for 24 hours. An 10ul pipette tip was used to create a wound on the surface of the adherent cells, and images were taken at 0 and 24 hours using a fluorescent inverted microscope.

7. Transwell invasion assay

20,000 cells resuspended in serum-free medium were added to the upper chamber of the transwell chamber, while 500 µl of 20% serum-containing medium was added to the lower chamber. After culturing for 24-36 hours, the unmigrated cells in the upper chamber were removed. The cells that migrated through the holes to the lower chamber were fixed with methanol, and then stained with 1% crystal violet and counted.

8. IHC
100 NSCLC tissue samples were stained with specific antibodies and bio-conjugated secondary antibodies, and then incubated with avidin-biotin-peroxide complex. Visualize the target protein using 3-amino-9-ethylcarbazole chromogen. Two experienced doctors in the pathology department scored the staining intensity of the IHC staining results: no staining was scored 0 point; weak staining was scored 1 point; medium staining was scored 2 points; strong staining was scored 3 points. Score based on the percentage of counted positive staining cells in the overall cells: <10%, 0 point; 10%-25%, 1 point; 26%-60%, 2 points; more than 60%, 3 points. Multiply the above two parameters, the obtained immune score was 0-9. A score of <4 was defined as low expression, and a score of ≥ 4 was defined as high expression.

9. Xenograft experiment

BABL/C nude mice were purchased from Guangdong Laboratory Animal Co. Ltd (Guangzhou, China). The animal experiments were conducted under the guidance of the Animal Research Ethics Committee of Nanjing Medical University. Six nude mice in each group were inoculated tumor cells suspension through the tail vein at a density of $1 \times 10^6$. The general condition of the nude mice was observed and recorded every three days. After 8 weeks of injection the lungs were taken out, and the metastases were observed and photographed. The removed lung tissues were fixed with formaldehyde. Tissue sections were made with paraffin, and stained with hematoxylin and eosin (HE).

10. Treatment for the patients

According to the clinical practice guidelines of oncology of National Comprehensive Cancer Network (NCCN), the treatment plan was developed. The 100 NSCLC patients included in the study were all stage I-IIIA. All the patients underwent radical resection of lung cancer, and the surgical plan was thoracoscopic one-way lobectomy plus mediastinal lymph node dissection. The excised tumor tissue specimens were subjected to histopathological examination to determine the tumor type, differentiation and pathological stage. Then according to the pathological stage, patients in stage Ib-IIIA received adjuvant chemotherapy for 4 cycles while patients in stage Ia did not. The adjuvant chemotherapy regimen is NP regimen (vinorelbine 25mg/m² for the 1st day and the 8th day plus cisplatin or nedaplatin 80mg/m² for the 1st day, every 21 days a cycle) or GP regimen (gemcitabine 1000mg/m², for the 1st day and the 8th day plus cisplatin or nedaplatin 80mg/m² for the 1st day, every 21 days a cycle).

11. Follow-up

Follow-up was conducted by telephone or outpatient service from the first day of treatment to death or the deadline for follow-up. After treatment, the patients were followed up every 3 months for the first 2 years, every six months from the 3rd to the 5th year, and every year after 5 years. Follow-up content included physical examination, hematology test, imaging examination, etc.
12. Statistical analysis

Data were presented as mean±standard deviation (SD), and SPSS 25.0 software was used for statistical analysis. The Kaplan-Meier method was used to calculate the cumulative survival time of clinical samples. Cox regression model was used to perform multivariate analysis on the parameters that were different in single factor analysis. Use chi-square test or t-test to compare the differences between the two groups. When $P$-value $<$ 0.05, the difference was considered to be statistically significant. Use Graphpad 5.0 software to draw the results.

Results

1. ZNF488 as an oncogene may play an important role in the progression of NSCLC

By searching the online databases of TCGA and GEPIA, we found that compared with normal lung tissues, ZNF488 was significantly higher in NSCLC tissues (Fig. 1. A). Compared with paired adjacent tissues, the expression level of ZNF488 in NSCLC tissues was significantly higher than that in adjacent tissues (Fig. 1. B-C). By analyzing the results of IHC in the HPA online database, we found that ZNF488 was highly expressed in NSCLC tissues compared with normal lung tissues (Fig. 2. A-D). Moreover, the five-year survival rate in ZNF488 high-expressing group was significantly lower than that low-expressing group (Fig. 1. D).

2. The expression of ZNF488 in NSCLC tissues and cell lines

We used qRT-PCR to detect the expression level of ZNF488 mRNA in 15 paired NSCLC tissues and adjacent normal lung tissues. The results showed that at mRNA level, the expression level of ZNF488 in NSCLC tissues was significantly higher than the adjacent lung tissues (Fig. 3. A). Western blot (Fig. 3. B) and IHC (Fig. 4) also showed the consistent results.

3. The relationship between the expression level of ZNF488 and clinical characteristics in NSCLC tissues

We analyzed the correlation between the expression level of ZNF488 and the clinical characteristics in 100 NSCLC tissues. The clinical data of two groups were comparable (Table 1). Univariate analysis showed that the expression level of ZNF488, T stage, N stage, TNM stage, distant metastasis, local recurrence and disease progression were prognostic factors for NSCLC patients ($p < 0.05$). Further multivariate analysis showed that the expression of ZNF488, TNM stage, and disease progression were independent prognostic factors in NSCLC patients ($p < 0.05$). The prognosis of the patients with high ZNF488 expression, high TNM staging and greater disease progression was poor (Table 2). We also used
the Kaplan-Meier analysis to cumulate the effects of ZNF488 on patients’s survival. The results showed that the 1, 3, and 5-year overall survival rates of 100 patients with stage I-IIIA NSCLC were 97.0%, 77.0%, 64.0% respectively (Fig. 5. A, Table 3). OS, LRFS, DMFS, PFS, stage I OS, and stage II OS of ZNF488 high expression group were lower than those of low expression group, and the difference was statistically significant ($p < 0.05$, Fig. 5. B-H, Table 4). The above results suggested that the expression level of ZNF488 had a significant correlation with the prognosis of NSCLC.
Table 1
Comparison of clinical data of ZNF488 low-expressing and high-expressing group

| Clinical Data     | Expression of ZNF488 | $\chi^2$ | $P$ value |
|-------------------|----------------------|----------|-----------|
|                   | Low or none, N = 58 (%) | High, N = 42 (%) |
| Age               | 35                   | 25       | 0.025     | 0.875     |
| > 60              | 25                   | 16       |           |           |
| $\leq$ 60         |                      |          |           |           |
| Gender            | 56                   | 40       | 0.109     | 0.741     |
| Male              | 2                    | 2        |           |           |
| Female            |                      |          |           |           |
| TNM Stage         | 25                   | 17       | 0.501     | 0.778     |
| I                 | 13                   | 12       |           |           |
| II                | 20                   | 13       |           |           |
| IIIA              |                      |          |           |           |
| T Stage           | 23                   | 16       | 0.347     | 0.841     |
| T1                | 16                   | 10       |           |           |
| T2                | 19                   | 16       |           |           |
| T3                |                      |          |           |           |
| N Stage           | 44                   | 26       | 2.314     | 0.314     |
| N0                | 11                   | 12       |           |           |
| N1                | 3                    | 4        |           |           |
| N2                |                      |          |           |           |
| KPS               | 48                   | 35       | 0.100     | 0.995     |
| $\geq$ 90        | 7                    | 5        |           |           |
| < 90 and $\geq$ 80 | 3                  | 2        |           |           |
| < 80 and $\geq$ 70 |                    |          |           |           |
Table 2
Univariate and multivariate analysis of overall survival in 100 cases

| Items                              | Univariate analysis |                 |           | multivariate analysis |                 |           |
|------------------------------------|---------------------|-----------------|-----------|-----------------------|-----------------|-----------|
|                                    | HR                  | 95% CI          | P value   | HR                    | 95% CI          | P value   |
| The expression level of ZNF488     | 2.633               | 1.366–4.999     | 0.003     | 2.264                 | 1.069–4.798     | 0.033     |
| (High VS Low)                      |                     |                 |           |                       |                 |           |
| Age                                | 1.161               | 0.612–2.202     | 0.648     | /                     |                 | /         |
| (>60 VS ≤ 60)                      |                     |                 |           |                       |                 |           |
| Gender                             | 1.590               | 0.383–6.592     | 0.523     |                       |                 |           |
| (Male VS Female)                   |                     |                 |           |                       |                 |           |
| T Stage                            | 1.428               | 1.089–1.874     | 0.010     | 0.785                 | 0.455–1.354     | 0.384     |
| (T1 VS T2 + T3)                    |                     |                 |           |                       |                 |           |
| N Stage                            | 3.640               | 2.221–5.965     | 0.000     | 1.362                 | 0.588–3.152     | 0.471     |
| (N0 VS N1 + N2)                    |                     |                 |           |                       |                 |           |
| TNM Stage                          | 2.820               | 1.870–4.253     | 0.000     | 3.008                 | 1.259–7.183     | 0.013     |
| (I + II VS IIIA)                   |                     |                 |           |                       |                 |           |
| Distant metastasis                 | 3.190               | 1.611–6.317     | 0.001     | 2.265                 | 0.929–5.521     | 0.072     |
| (Yes VS No)                        |                     |                 |           |                       |                 |           |
| Local recurrence                   | 2.455               | 1.221–4.934     | 0.012     | 0.465                 | 0.200–1.081     | 0.075     |
| (Yes VS No)                        |                     |                 |           |                       |                 |           |
| Progression                        | 12.544              | 5.483–28.743    | 0.000     | 7.113                 | 2.608–19.403    | 0.000     |
| (Yes VS No)                        |                     |                 |           |                       |                 |           |

Table 3
OS of 100 patients

| OS(Year) | 1    | 2    | 3    | 4    | 5    |
|----------|------|------|------|------|------|
| Survival rate | 97.0% | 89.0% | 77.0% | 67.0% | 64.0% |
Table 4
Statistics of survival analysis of the two groups

| Item                          | X²  | P    |
|-------------------------------|-----|------|
| Comparison of the two groups of OS | 6.031 | 0.014 |
| Comparison of the two groups of LRFS | 7.574 | 0.006 |
| Comparison of the two groups of DMFS | 7.771 | 0.005 |
| Comparison of the two groups of PFS | 7.494 | 0.006 |
| Comparison of the two groups of stage I OS | 3.932 | 0.047 |
| Comparison of the two groups of stage II OS | 4.899 | 0.027 |
| Comparison of the two groups of stage III OS | 2.175 | 0.140 |

4. Overexpression of ZNF488 promoted the invasion and metastasis of NSCLC cells in vivo and in vitro

The NSCLC cell lines NCI-H520 and PC-9 were used to establish ZNF488 over-expressing stable cell lines and vector control cell lines. The Western blot showed that the ZNF488 stable over-expressing cell lines were successfully constructed (Fig. 6. A). The wound healing assay showed that the cell migration of NCI-H520 and PC-9 cells increased after overexpression of ZNF488 (Fig. 6. B). Transwell invasion test showed that the invasion ability of NCI-H520 and PC-9 cells was enhanced after overexpression of ZNF488 (Fig. 6. C).

Tumor cells were injected into the tail vein of nude mice to observe the number of lung metastases. The results showed that the number and volume of lung metastases in ZNF488 over-expressing group were significantly higher than those in vector (Fig. 6. D-E). We also performed HE staining to verify the reliability of metastases (Fig. 6. F-G).

5. ZNF488 induces EMT in NSCLC cells to promote invasion and metastasis.

After overexpression of ZNF488, the expression levels of epithelial marker proteins E-Cadherin and α-Catenin decreased at the transcriptome level, and the interstitial marker proteins Fibronectin and Vimentin increased (Fig. 7. A). The test at the protein level presented the consistent results (Fig. 7. B). The above results indicated that overexpression of ZNF488 can induce EMT in NSCLC.

6. MAPK/ERK signaling pathway may play an important role in ZNF488-mediated high invasion and metastasis
We performed gene chip detection on ZNF488 over-expressing cell lines and vector. The results showed that a total of 233 genes with a multiple of more than twice the difference. Among these genes, 138 were up-regulated and 95 were down-regulated. The online database DAVID was used to analyze the differential genes in the KEGG pathway, and the results showed that the MAPK signal pathway played an important role in ZNF488 in promoting the invasion and metastasis of NSCLC cells (Table 6). Western blot showed after over-expressing ZNF488, the expression of p-ERK1/2, MMP2 and MMP9 increased, but t-ERK1/2 did not change significantly (Fig. 8).

| Category       | Term                                      | $P$ value |
|----------------|-------------------------------------------|-----------|
| Kegg Pathway   | MAPK signaling pathway                    | 0.03      |
| Kegg Pathway   | Pathways in cancer                        | 0.005     |
| Kegg Pathway   | Cytokine-cytokine receptor interaction    | 6.42E-04  |
| Kegg Pathway   | B cell receptor signaling pathway         | 0.01      |

7. MEK inhibitor AZD6244 reversed the invasion and migration ability of NSCLC enhanced by overexpression of ZNF488

In order to deeply explore the role of MAPK / ERK signaling pathway in the promotion of invasion and metastasis of NSCLC by ZNF488, we carried out rescue experiments and designed 3 experimental groups: control group (NC group), ZNF488 over-expressing group and AZD6244 + ZNF488 group. Wound healing assay showed that overexpression of ZNF488 can promote the migration of NSCLC cells (NC group vs. ZNF488 over-expressing group), and AZD6244 can partially reverse the promotion of ZNF488 on the migration of NSCLC cells (NC group VS AZD6244 + ZNF488 group). Transwell invasion assay also proved that AZD6244 can partially reverse the enhancement effect of ZNF488 on the invasion ability of NSCLC cells (Fig. 9). The above results indicated that AZD6244 can inhibit the invasion and migration of NSCLC by inhibiting the activation of MAPK/ERK signaling pathway by ZNF488.

8. MEK inhibitor AZD6244 reversed the EMT process induced by ZNF488

We used Western blot to explore whether the MEK inhibitor AZD6244 can antagonize the EMT process induced by ZNF488. The experiment is divided into 3 groups: NC group, ZNF488 group, and AZD6244 + ZNF488 group. The results showed that compared with the NC group, the ZNF488 group can inhibit the expression of E-cadherin and up-regulate the expression of Vimentin, Fibronectin and p-ERK1/2. However, when MEK inhibitor AZD6244 was added, the expression levels of Vimentin, Fibronectin, and p-ERK1/2 in
the AZD6244 + ZNF488 group were suppressed compared with the ZNF488 group, while the expression level of E-cadherin was partially restored (Fig. 10).

In summary, as an independent prognostic indicator, ZNF488 promoted the EMT process of NSCLC cells by activating the MAPK/ERK signaling pathway.

Discussion

As a member of the zinc finger protein family, ZNF488 has biological functions involved in regulating gene expression and cell differentiation\[^{25}\], but its function and molecular mechanism in NSCLC have not been reported so far. Therefore, we explored the possibility of ZNF488 as a potential tumor marker in the progression of NSCLC.

We used the lung cancer data provided by TCGA to conduct a differential gene analysis. The results showed ZNF488 was significantly highly expressed in NSCLC tissues. Further analysis of paired data showed the expression level of ZNF488 in NSCLC tissues was also significantly higher than that in adjacent tissues. Subsequently, we used the IHC results in the HPA database to analyze the difference in the expression of ZNF488 in NSCLC and normal lung tissues. The results showed that ZNF488 is significantly highly expressed in NSCLC tissues, but was basically not expressed in normal lung tissues. We further analyzed the clinical data of patients with NSCLC in the TCGA database. The results showed the five-year survival rate of patients with high ZNF488 expression was significantly lower than that of low expression. The above results showed that ZNF488 was highly expressed in NSCLC tissues and was closely related to the poor prognosis of patients.

In order to verify the analysis results of the database, we used qRT-PCR to analyze the expression of ZNF488 in 15 pairs of NSCLC and their corresponding adjacent tissues. The results showed that ZNF488 was significantly higher in NSCLC tissues compared with adjacent tissues. We further expanded the clinical tissue sample size to 100 cases, used IHC to stain the collected clinical samples, and divided the tissue samples into ZNF488 high expression group and low expression group, and then analyzed the correlation of clinical data of patients and the expression level of ZNF488. We further expanded the clinical tissue sample size to 100 cases and performed IHC staining. According to the results, the tissue samples were divided into ZNF488 high-expressing group and low-expressing group. Then the correlation between the patient's clinical characteristics and the expression level of ZNF488 was analyzed. Univariate analysis showed that the expression level of ZNF488, T stage, N stage, TNM stage, distant metastasis, local recurrence and disease progression were prognostic factors for NSCLC. Multivariate analysis showed that the expression of ZNF488, TNM stage and disease progression were independent prognostic factors for NSCLC. The prognosis of NSCLC with high ZNF488 expression, high TNM stage and greater disease progression was worse. Survival analysis showed that the 1, 3, and 5-year overall survival rates of 100 cases of stage I-IIIA NSCLC were 97.0%, 77.0%, and 64.0% respectively, which were similar to the survival rates of stage I-IIIA NSCLC in most studies \[^{3-7}\]. The OS, LRFS, DMFS, PFS, stage I OS, and stage II OS of the ZNF488 high-expressing group were lower than those of the low-expressing group. Although
there was no statistical difference in the stage III OS between the two groups, the survival curve showed the same trend as the above results, which was considered to be related to the small sample size. The above results showed that the expression level of ZNF488 had a significant correlation with the prognosis of patients in NSCLC.

For further explore the molecular mechanism, we carried out the experiments in vivo and in vitro. Western blot showed that the expression of ZNF488 in NSCLC cells was generally higher than that in normal lung epithelial cells. We chose NCI-H520 and PC-9 cell lines with relatively low expression of ZNF488 to construct cell lines that stably over-expressing ZNF488. The wound healing assay showed that overexpression of ZNF488 can promote the healing of cell wounds. Transwell invasion assay showed that overexpression of ZNF488 can enhance the invasion of NSCLC cells. Xenograft experiment showed that the number and volume of lung metastases after overexpression of ZNF488 were significantly higher than those in vector.

Tumor invasion and metastasis is a multi-step, continuous and complex process involving multiple genes, and EMT plays an important role in it\cite{26,27}. Scholars have found that extracellular signals can bind to receptors and transmit corresponding signals to cells for activate some transcription factors, which ultimately regulate the EMT process of lung cancer\cite{28}. EMT can be used as an indicator of tumor treatment sensitivity and prognosis to a certain extent\cite{29–32}. It has been confirmed that multiple signal transduction pathways such as TGF-β, PI3K/AKT, RAS-MAPK, etc. participate in this process in cells\cite{33–35}. When the signaling pathway is activated, transcription factors change. The change cause the expression of epithelial markers such as E-calcin in tumor cells to decrease, while the expression levels of mesenchymal markers such as vimentin and N-calcin increase\cite{36–40}. At the same time, the tight junctions between cells are weakened, and the invasion and migration capabilities are enhanced\cite{41–43}.

In this study, Western blot showed that ZNF488 can inhibit the expression of epithelial markers E-Cadherin and α-Catenin, while the expression of mesenchymal markers Fibronectin and Vimentin increased. This suggested that ZNF488 can induce EMT in NSCLC cells. Subsequently, we analyzed the differentially changed genes between the ZNF488 over-expressing group and vector through gene chips, and performed KEGG analysis and Western blot verification of the differential genes. The results showed that ZNF488 might exert its biological function by activating the MAPK/ERK signaling pathway. In order to further verify our conjecture, we added MEK inhibitor AZD6244 to the ZNF488 over-expressing cells. The results showed that compared with the ZNF488 over-expressing group, the AZD6244 + ZNF488 group can significantly reduce cell invasion and migration, and inhibit the expression of interstitial markers Fibronectin and Vimentin. The above results suggested that ZNF488 can induce the EMT process of NSCLC cells by activating the MAPK/ERK signaling pathway, and promoted the invasion and metastasis.

In summary, this study found for the first time that ZNF488 was significantly highly expressed in NSCLC tissues, and its expression level was closely related to the prognosis of patients. In terms of mechanism, ZNF488 can induce EMT in NSCLC cells by activating the MAPK/ERK signaling pathway.
Conclution
In summary, our study demonstrated that ZNF488 as an independent prognostic indicator, can promote the invasion and metastasis by activating the MAPK/ERK signaling pathway in NSCLC, which may represent a novel therapeutic target for NSCLC.

Abbreviations
ZNF488: zinc finger protein 488; NSCLC: non-small cell lung cancer; qRT-PCR: Quantitative Reverse transcription polymerase chain reaction; IHC: immunohistochemical staining; OS: overall survival; LRFS: local recurrence-free survival; DMFS: distant metastasis-free survival; PFS: and progression-free survival; EMT: epithelial-mesenchymal transition; AJCC: American Joint Committee on Cancer; HE: hematoxylin and eosin; NCCN: National Comprehensive Cancer Network; SD: standard deviation.

Declarations
1. Ethics approval and consent to participate
All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethics Committee of the Institutional Ethical Review Board of Jiangsu Cancer Hospital. All patients studied signed an informed consent for participation. All animal procedures and care were conducted in accordance with institutional guidelines and in compliance with national and international laws and policies.

2. Consent for publication
Not applicable.

3. Availability of data and materials
The datasets used and analysed during the current study are available from the corresponding authors on reasonable request.

4. Competing interests
The authors declare that they have no competing interests.

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6. Authors’ contributions

YZ and HZ wrote the paper; YZ, DW, DZ and XH conceived and designed the study; YZ, HZ, NJ, JG and LW did the experiments; LZ, CC, YG and JF collect and analyzed the data. All authors read and approved the final manuscript.

7. Acknowledgements

Not applicable

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Figures
TCGA database search results showed that ZNF488 was highly expressed in NSCLC tissues and was closely related to poor prognosis. (A) qRT-PCR to detect the expression of ZNF488 in unpaired NSCLC tissues and normal lung tissues; (B) qRT-PCR to detect the expression of ZNF488 in matched NSCLC tissues and adjacent tissues; (C) qRT-PCR to detect the expression of ZNF488 in matched NSCLC tissues and adjacent tissues, and normalized to adjacent tissues; (D) Survival analysis of ZNF488 high-expressing group and low-expressing group.

Figure 2
IHC results in the HPA online database. (A) Expression of ZNF488 in normal lung tissues; (B) Expression of ZNF488 in NSCLC tissues; (C) Partial enlargement of picture A; (D) Partial enlargement of picture B.
Figure 3

The expression of ZNF488 in NSCLC tissues and cell lines. (A) qRT-PCR to detect the expression of ZNF488 in paired NSCLC tissues and adjacent normal lung tissues, GAPDH was the internal control; (B) Western blot to detect the expression of ZNF488 in NSCLC cell lines.** means p<0.01.

Figure 4

Representative images of IHC to detect the expression of ZNF488 in NSCLC tissues. (A) Negative ZNF488 staining with normal rabbit IgG; (B) Weak staining; (C) Weak to moderate staining; (D) Moderate staining; (E) Strong staining.
Figure 5

The relationship between ZNF488 expression and survival in NSCLC. (A) OS of 100 patients; (B) Comparison of two groups of OS; (C) Comparison of two groups of PFS; (D) Comparison of two groups of LRFS; (E) Comparison of two groups of DMFS; (F) Comparison of two groups of stage I OS; (G) Comparison of two groups of stage II OS; (H) Comparison of two groups of stage III OS.
Figure 6

Overexpression of ZNF488 promoted the invasion and metastasis ability of NSCLC cells; (A) Western blot detection of ZNF488 protein over-expressing in stable expression cell lines; (B) Wound healing assay to detect the effect of overexpression of ZNF488 on the migration ability of NCI-H520 and PC-9 cells; (C) Transwell invasion test to detect the effect of overexpression of ZNF488 on the invasion ability of NCI-H520 and PC-9 cells; (D) Formation of lung metastases after tail vein injection of stable over-expressing
ZNF488 and vector group cell lines in nude mice; (E) Statistical analysis of the number of lung metastases in the ZNF488 over-expressing group and vector; (F) HE staining of normal lung tissue; (G) HE staining of lung metastases. ** means p<0.01.

Figure 7

Overexpression of ZNF488 induced EMT in NSCLC cells. (A) qRT-PCR detected the expression of E-Cadherin, α-Catenin, Fibronectin and Vimentin; (B) Western blot was used to detect the expression of E-cadherin, α-Catenin, Fibronectin and Vimentin. ** means p<0.01.
Figure 8

The MAPK/ERK signaling pathway played an important role in the high invasion and metastasis mediated by ZNF488. (A) The results of gene chip analysis of the vector and ZNF488 over-expressing group; (B) Western blot showed that the expression of p-ERK1/2, MM2, and MMP9 increased, while t-ERK1/2 did not change significantly.
MEK inhibitor AZD6244 reversed the invasion and migration ability of NSCLC enhanced by overexpression of ZNF488. (A) Wound healing assay detected the effect of adding MEK inhibitor ADZ6244 to cells over-expressing ZNF488 on migration ability; (B) Transwell invasion assay detected the effect of adding MEK inhibitor ADZ6244 to cells over-expressing ZNF488 on invasion ability. ** means p<0.01.
MEK inhibitor AZD6244 reversed the EMT process induced by ZNF488.