# The upregulation of TMPRSS4, partly ascribed to the downregulation of miR-125a-5p, promotes the growth of human lung adenocarcinoma via the NF-κB signaling pathway

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**Abstract.** In this study, with the aid of microarray technology, transmembrane protease serine 4 (TMPRSS4), a novel member of the serine protease family, was found to be upregulated in the majority of lung adenocarcinoma (LUAD) tissues compared to normal lung tissues. Of note, the clinical significance of TMPRSS4 in LUAD has not yet been reported, at least to the best of our knowledge. Through immunohistochemistry assays, we found that TMPRSS4 was overexpressed in LUAD tissues and that the TMPRSS4 expression level was also proportionally associated with the AJCC clinical stage, T stage and pathological grade. Moreover, a high expression of TMPRSS4 was found to be associated with adverse outcomes and was a significant independent factors predicting a poor prognosis. To elucidate the possible mechanisms responsible for the overexpression of TMPRSS4, we examined at microRNAs (miRNAs or miRs), which are small non-coding RNAs commonly dysregulated in human malignancies and are known to promote carcinogenesis by interacting with other types of RNAs. By means of bioinformatics analysis, a miRNA potentially targeting TMPRSS4 mRNA, namely miR-125a-5p, was selected. Dual luciferase reporter gene assays were then performed to verify the interaction. The results of MTT assays and apoptotic assays revealed that miR-125a-5p significantly inhibited cell growth and enhanced apoptosis, and the silencing of TMPRSS4 had similar effects. Furthermore, we observed that either the overexpression of miR-125a-5p or the silencing of TMPRSS4 prevented the activation of the nuclear factor (NF)-κB signaling pathway. On the whole, our findings illustrate that TMPRSS4 may be a candidate oncogene and may thus serve as a prognostic biomarker for LUAD, and its overexpression may be partly ascribed to the downregulation of miR-125a-5p. The dysregulation of miR-125a-5p and TMPRSS4 affect the biological function of LUAD cells via the NF-κB signaling pathway. The miR-125a-5p/TMPRSS4/NF-κB axis may thus provide novel insight into the pathogenic mechanisms of LUAD and may be used in the development of novel treatment strategies for LUAD.

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*Key words:* microRNA-125a-5p, transmembrane protease serine4, nuclear factor-κB signaling pathway, lung adenocarcinoma, growth, prognosis

## Introduction

Lung cancer is the most common cause of cancer-related mortality worldwide and lung adenocarcinoma (LUAD) is the most common lung cancer type and is associated wiht a poor prognosis (1,2). Although novel therapies, such as targeted chemotherapy or immunological therapy, provide more strategies for the treatment of LUAD, the survival rate still remains low (3). Hence, the exploration of novel targets for LUAD is required.

In this study, with the development of microarray technology, we analyzed a microarray of LUAD in Gene Expression Omnibus and found out the differential expression genes. Transmembrane protease serine 4 (TMPRSS4), which is a membrane bound protein with serine protease activity (4), was selected for our analysis. TMPRSS4 has been shown to be overexpressed in several types of cancer, such as pancreatic carcinoma and lung carcinoma (5,6). A previous study indicated that the upregulation of TMPRSS4 was associated with adverse outcomes and promoted invasion and metastasis in lung squamous cell carcinoma (7). However, to date, there is only limited information available on the association between TMPRSS4 and the clinical characteristics of LUAD.

microRNAs (miRNAs or miRs) are known to have bright prospects for use in the development of novel targeted therapies and the prognostic prediction of human lung cancers (8). It is still unclear as to which miRNAs potentially target TMPRSS4 in LUAD. In this study, by bioinformatics analysis, miR-125a-5p was identified, which is a potential

miRNA targeting TMPRSS4 mRNA. As has been reported, miR-125a-5p is a key regulator in carcinogenesis as it is expressed at abnormal levels in specific types of cancer, such as colorectal cancer (9), pancreatic cancer (10) and prostate cancer (11). Studies have demonstrated that miR-125a-5p is expressed at low levels in non-small cell lung cancer (NSCLC) tissues and cell lines, and is associated with cancer progression and a poor prognosis (12,13). To date, the role of miR-125a-5p in lung carcinogenesis has not yet been elucidated fully, and the association between miR-125a-5p and TMPRSS4 mRNA has not yet been investigated, at least to the best of our knowledge. In the present study, the expression level of TMPRSS4 in LUAD and its potential clinical significance were investigated by immunohistochemistry (IHC) assays, Spearman's correlation analysis and survival analysis. The interaction between TMPRSS4 mRNA and the 3'-UTR of miR-125a-5p was confirmed by dual luciferase assays. The effect of the neutralization of TMPRSS4 and the ectopic expression of miR-125a-5p in LUAD cell lines were also assessed. Furthermore, we partly explored the mechanisms through which miR-125a-5p and TMPRSS4 affect the biological function.

## Materials and methods

*Microarray data and data processing*. The Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) contains freely available microarray and next-generation sequencing data. The GSE43458 and GPL6244 datasets were obtained from the GEO database. The detailed information of the microarray has been described previously (14). The R program was applied to screen differentially expressed genes (DEGs) between the LUAD and normal samples. The adjusted P-values (adj. P-value) were used to correct false-positive results. The screening criteria were set as adj. P<0.01 and a fold change >4.

*Tissue microarrays and IHC*. In total, 101 pairs of human LUAD tissues and adjacent normal lung tissues (2-3 cm away from the edge of the tumor, which was confirmed to have no residual malignancy by pathological sectioning) were obtained from the tissue specimens bank of the Department of Thoracic Surgery of The First Hospital of China Medical University, Shenyang, China. All the patients included had not received neoadjuvant therapy prior to surgery. The follow-up data of the 101 cases were cut-off in August 2016, and the overall survival was defined as the day of surgery to the day of death or to the last follow-up. All the patients signed informed consent forms for the use of their samples in scientific research. The experiments were approved by the Ethics Committee of the First Hospital of China Medical University (IRB Approval 2012-40-2). The tissue microarrays and IHC were performed by Shanghai Outdo Biotech Co. Ltd. (Shanghai, China), of which the detailed processes have been previously described by Ding *et al* (15). The TMPRSS4 antibody (concentration, 20 $\mu$g/ml; ab188816; Abcam, Cambridge, MA, USA) and the EnVision ™+ detection system (Dako, Glostrup, Denmark) were used according to the manufacturer's instructions. Immunostained microarrays were scored by multiplying the intensity (0-3 score) and extent of staining (0-25%, score 1; 25-50%, score 2; 50-75%, score 3; and 75-100%, score 4) for each tissue point as previously described by Chen *et al* (16). A total score of <3.5 was regarded as a low expression, or otherwise, a high expression.

*Cell culture*. The human LUAD cell lines, NCI-H358 and A549, were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured according to the manufacturer's instructions. Although the NCI-H358 cell line is considered that it may be identical to the NCI-H358M cell line as (https://web.expasy.org/cellosaurus/CVCL_1559), we considered that this would not affect the outcomes of our study.

*Identification of potential miRNAs targeting TMPRSS4*. Three miRNA-target gene databases, miRanda, TargetScan (Release 6.2) and MicroCosm 5 were used to predict the miRNAs which suppress the expression of TMPRSS4. From the intersection of these miRNAs, we identified as the potential miRNAs targeting TMPRSS4.

*Oligonucleotide synthesis and transient transfection*. All the material used was purchased from GenePharma (Shanghai, China), including small interfering RNA (siRNA), overexpression plasmid vectors with pcDNA 3.1, miRNA inhibitors, miRNA mimics and respective negative control (NC). The sequences of the miRNA mimics were as follows: 5'-UCCCUG AGACCCUUUAACCUGUGA-3' and 5'-ACAGGUGAGGU UCUUGGGAGCC-3'; the sequences of the miRNA inhibitors were 5'-UCACAGGUUAAAGGGUCUCAGGGA-3'; the sequence of NC (for both mimics and inhibitors) was 5'-CAG UACUUUUGUGUAGUACAA-3'. The sequence of the siRNA was 5'-CCTGGCGAGTATCATCATT-3'. The transfected amount of oligonucleotides were 75 pmol per well in a 6-well plate, and the transfected amount of plasmids was 2.5 $\mu$g. Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) was utilized to conduct transient transfection according to the manufacturer's instructions. At 48 h after transfection, the cells were harvested to perform the assays.

*RNA extraction and reverse transcription-quantitative (real-time) PCR (RT-qPCR)*. Total RNA was extracted from the NCI-H358 and A549 cells usung TRIzol reagent (Takara, Beijing, China). Total RNA was reverse transcribed into single-strand cDNA using the First Strand cDNA Synthesis kit (Thermo Scientific, Waltham, MA, USA). qPCR was performed using SYBR-Green Master Mix (Roche, Rotkreuz, Switzerland) using an ABI 7500 Real-Time PCR system (Applied Biosystems, Waltham, MA, USA) and the parameters were set according to instructions provided with SYBR-Green Master Mix. β-actin was used as an internal control for TMPRSS4. The primers were synthesized by Ribobio Co. (Guangzhou, China) and the primer sequences of TMPRSS4 mRNA as well as β-actin have been described previously (17). The reverse transcription PCR and RT-qPCR analysis of miR-125a-5p was performed according to the method and primers previously described by Jia *et al* (10). The relative expression value was calculated by the $2^{-\Delta\Delta Cq}$ method (18). For the ease of comparison, values for cells without any treatment (blank group) were set equal to 1.

*Protein extraction and western blot analysis*. The cells were lysed and the protein was extracted with RIPA

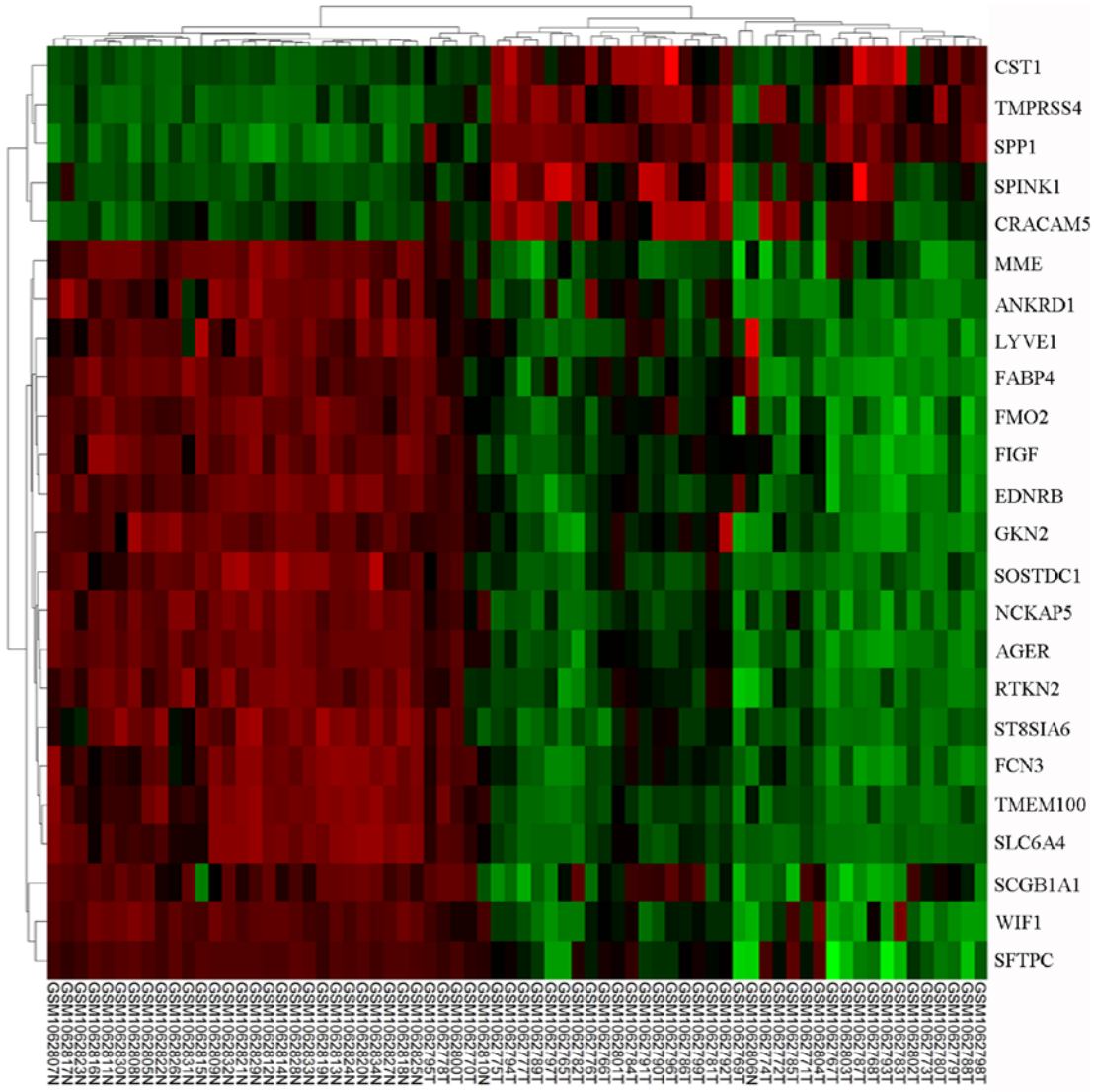


Figure 1. Bioinformatics analysis. Cluster analysis for the differentially expressed genes between lung adenocarcinoma tissues (GSM serial number with 'T') and paired adjacent tissues (GSM serial number with 'N'). Twenty-four DEGs are highlighted in green (downregulation) and red (upregulation).

solution supplemented with protease inhibitors and PMSF (Beyotime, Beijing, China). Total proteins were extracted by centrifugation at 12,000 x g at 4˚C for 20 min the protein concentration was determined by the BCA Protein assay kit (Beyotime). Subsequently, 40 $\mu$g of total proteins were separated by 10% SDS-PAGE and followed by transfer onto 0.45 $\mu$m nitrocellulose membrane. The membranes were blocked with Tris-buffered saline containing 0.5% Tween-20 supplemented with 5% defatted milk for 1.5 h. Subsequently, the membranes were incubated with primary antibody for 14-16 h at 4˚C. Finally, the membranes were washed and incubated with secondary antibody for 2 h at room temperature. According to the protocols, the antibodies used were as follows: primary antibodies against TMPRSS4 (concentration, 2 $\mu$g/ml; ab82176), nuclear factor (NF)-κB p65 (ab16502), Bcl-2 (ab32124), histone H3 (ab11946), p-IκBα (ab92700) and IκBα (ab32518) (all 1:500). The anti-β-actin antibody (ab6276) was diluted 1:5,000. The secondary antibody used was the HRP-labeled secondary antibody (diluted, 1:5,000; ab6741). All the antibodies were purchased from Abcam. Bands were observed by enhanced chemiluminescence (ECL) Plus Western blot detection reagents (Thermo Scientific).

*Dual luciferase assays*. To confirm whether miR-125a-5p can interact with TMPRSS4 mRNA, the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) pGLO-TMPRSS4 (wild-type and mutant-type) of miR-125a-5p was constructed by GeneChem (Shanghai, China). After culturing the NCI-H358 and A549 cells in 24-well plates overnight, the cells were co-transfected with miR-125a-5p mimics or NC, luciferase reporter plasmids and *Renilla* vector (pRL-TK) (GeneChem) at a ratio of 10:1 using Lipofectamine 2000. Luciferase activity was measured after 48 h by the Dual-Luciferase® Reporter assay system (Promega), according to the manufacturer's instructions, and the activity was normalized to the corresponding *Renilla* luciferase activity. For ease of comparison, values for cells with NC + mutant type 3'UTR (mt 3'-UTR) group were set equal to 1.

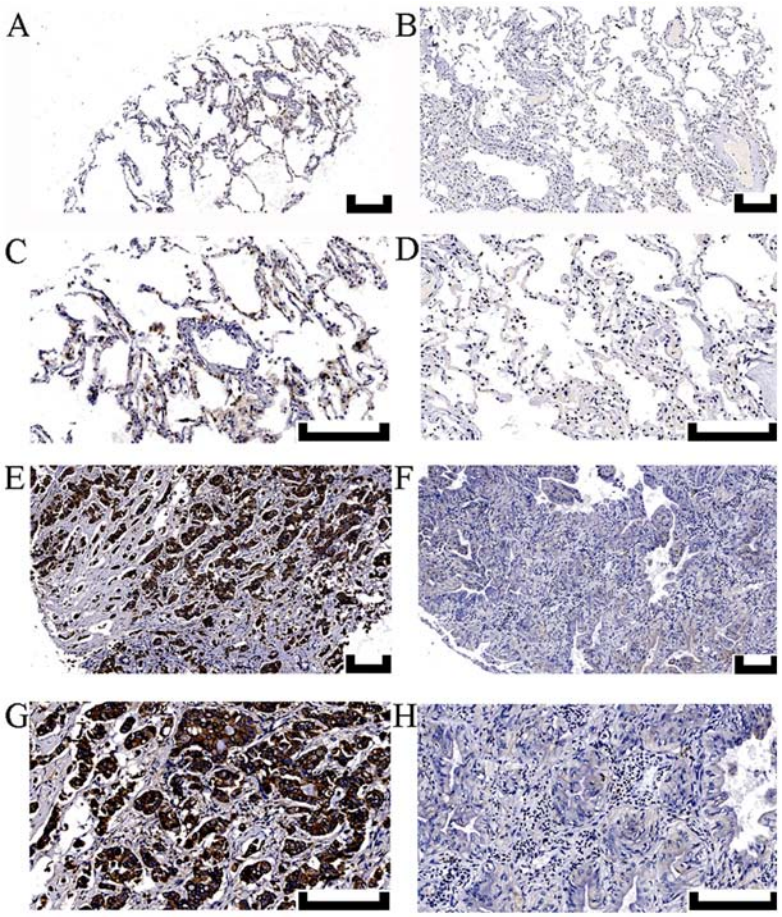


Figure 2. Immunostaining of TMPRSS4 protein. High expression level in lung tissues (A) magnification, x100 and (C) magnification, x200; low expression level in TMPRSS4 in lung tissues (B) magnification, x100 and (D) magnification, x200; high expression in lung adenocarcinoma (LUAD) tissues (E) magnification, x100 and (G) magnification, x200; low expression level in TMPRSS4 in LUAD tissues (F) magnification, x100 and (H) magnification, x200. Scale bar, 100 $\mu$m.

*MTT assays*. The transfected NCI-H358 and A549 cells were plated into 96-well plates at a density of $5x10^3$ cells each well, and cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kits (Beyotime) according to the protocol. Following the addition of 10 $\mu$l/well MTT (5 mg/ml) solution, the cells were incubated at 37˚C for 4 h. The absorbance at 570 nm was read using a microplate reader (Bio-Rad, Hercules, CA, USA) to evaluate the number of living cells.

*Apoptosis assays*. The transfected cells were seeded in 6-well plates and then collected by trypsin, respectively after 48 h. Flow cytometry (C6 type flow cytometer, BD Biosciences, San Jose, CA, USA) and the Annexin V-FITC/PI Apoptosis Detection kit (KeyGen Biotech, Nanjing, China) were used to detect the apoptosis on the basis of manufacturer's instructions. Each experiment was performed 3 times repeatedly.

*Statistical analyses*. The limma package in the R program was applied to search DEGs. SPSS 23.0 software and GraphPad prism 5.0 was utilized for statistical analyses. All data are presented as the means ± standard deviations (SD). The Chi-square ($\chi^2$) test, Spearman's correlation analysis, Kaplan-Meier curve and Cox hazard regression were applied to estimate the association between the expression level of TMPRSS4 and clinical characteristics. A Student's t-test and one-way analysis of variance (ANOVA; for multiple comparisons) with the SNK-q test. All the *in vitro* assays were repeated at least 3 times. A value of $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Identification of DEGs*. A total of 24 DEGs were identified from the GSE43458 dataset on the basis of adj. $P<0.01$ and |fold change| >4 (Fig. 1). Among these, 5 DEGs were found to be significantly overexpressed in LUAD, and TMPRSS4, a gene contributing to oncogenesis in NSCLC, was selected as our target gene (17,19).

*TMPRSS4 is highly expressed in LUAD tissues*. In total, 12 pairs of LUAD tissues and their adjacent lung tissues were excluded as they contained an off-targeted site, namely the tissues were not able to be stained. The expression of TMPRSS4 in the paired LUAD and lung tissues is shown in Fig. 2 and Table I. According to the results of IHC analysis, TMPRSS4 was highly expressed in the LUAD tissues and was expressed at low levels in the normal lung tissues ($P<0.05$). Spearman's rank correlation analysis was employed to describe the correlation between TMPRSS4 expression and grade, T-stage, N stage and TNM stage (Table II) as they contain ordinal dependent and

Table I. Differential expression of TMPRSS4 in paired tissue samples.

| Tissue sample | No. | TMPRSS4 expression High (%) | TMPRSS4 expression Low (%) | Chi-square value | P-value |
|---|---|---|---|---|---|
| Lung adenocarcinoma | 89 | 59 | 30 | 8.2161 | 0.004[a] |
| Pericancerous tissues | 89 | 40 | 49 | | |

[a]Statistically significant (P<0.05).

Table II. Correlation between TMPRSS4 expression and clinicopathological characteristics.

| Characteristic | TMPRSS4 expression High | TMPRSS4 expression Low | Total | $r_s$ | P-value |
|---|---|---|---|---|---|
| Grade | | | | 0.239 | 0.024[a] |
| 1 | 8 | 10 | 18 | | |
| 2 | 42 | 18 | 60 | | |
| 3 | 9 | 2 | 11 | | |
| T stage | | | | 0.226 | 0.033[a] |
| T1 | 8 | 10 | 18 | | |
| T2 | 33 | 15 | 48 | | |
| T3 | 13 | 3 | 16 | | |
| T4 | 5 | 2 | 7 | | |
| N stage | | | | 0.195 | 0.067 |
| N0 | 24 | 20 | 44 | | |
| N1 | 21 | 3 | 24 | | |
| N2 | 9 | 7 | 16 | | |
| N3 | 5 | 0 | 5 | | |
| TNM stage | | | | 0.255 | 0.016[a] |
| I | 17 | 17 | 34 | | |
| II | 16 | 5 | 21 | | |
| III | 23 | 8 | 31 | | |
| IV | 3 | 0 | 3 | | |

[a]Statistically significant (P<0.05).

independent variables with 3 or more groups. The Chi-square test was used to examine the association between TMPRSS4 expression and sex, age and M stage (Table III). The results revealed that a higher TMPRSS4 expression was associated with a higher AJCC clinical stage ($r_s$=0.255, P<0.05), T stage ($r_s$=0.226, P<0.05) and pathological grade ($r_s$=0.239, P<0.05). According to the follow-up visits, a Kaplan-Meier plotter and log-rank statistical test indicated that a higher TMPRSS4 expression was associated with adverse outcomes (Fig. 3A,

Table III. Association between TMPRSS4 expression and clinicopathological characteristics.

| Characteristic | Variables | TMPRSS4 expression High | TMPRSS4 expression Low | Total | $\chi^2$ | P-value |
|---|---|---|---|---|---|---|
| Age | ≤60 | 33 | 11 | 44 | 2.953 | 0.086 |
| | >60 | 26 | 19 | 45 | | |
| Sex | Male | 33 | 14 | 47 | 0.685 | 0.408 |
| | Female | 26 | 16 | 42 | | |
| M stage | M0 | 56 | 30 | 86 | 1.579 | 0.209 |
| | M1 | 3 | 0 | 3 | | |

Table IV. Multivariate analyses of the factors associated with the overall survival of patients with lung adenocarcinoma.

| Characteristic | Multivariate analysis HR | 95% CI | P-value |
|---|---|---|---|
| Expression | 3.253 | 1.506-7.027 | 0.003[a] |
| Sex | 0.787 | 0.418-1.481 | 0.458 |
| Age | 0.466 | 0.238-0.912 | 0.026[a] |
| T stage | 1.716 | 0.957-3.077 | 0.070 |
| N stage | 0.951 | 0.521-1.735 | 0.870 |
| M stage | 1.745 | 0.204-14.911 | 0.611 |
| TNM stage | 1.002 | 0.452-2.222 | 0.995 |
| Grade | 0.634 | 0.365-1.099 | 0.105 |

[a]Statistically significant (P<0.05).

P<0.05). Cox multivariate regression analysis revealed that TMPRSS4 expression and age were considered as independent significant prognostic factors of LUAD, respectively (Table IV).

*TMPRSS4 affects the proliferation and apoptosis of LUAD cell lines*. To further examine the effects of TMPRSS4 overexpression, the LUAD cell lines, NCI-H358 and A549, were selected to perform experiments at a cellular level as TMPRSS4 is highly expressed in NCI-H358 cells and is expressed at low levels in A549 cells, as previously reported by Nguyen *et al* (20). The cells in the si-TMPRSS4, the TMPRSS4 overexpression plasmid vector and the negative control groups were transfected, respectively in 6-well plates and MTT assays were then employed to measure cell proliferation. The transfection efficiency was first confirmed by RT-qPCR (Fig. 3B; P<0.05). The results revealed that the silencing of TMPRSS4 significantly inhibited cell proliferation compared with the control groups, and the overexpression of TMPRSS4 achieved the opposite effect (Fig. 4A, P<0.05). Moreover, cell apoptosis assays were conducted with the transfected cells by using the Annexin V-FITC/PI Apoptosis Detection kit. The results revealed that the apoptotic percentage of cells in the si-TMPRSS4 group was significantly higher than that in the

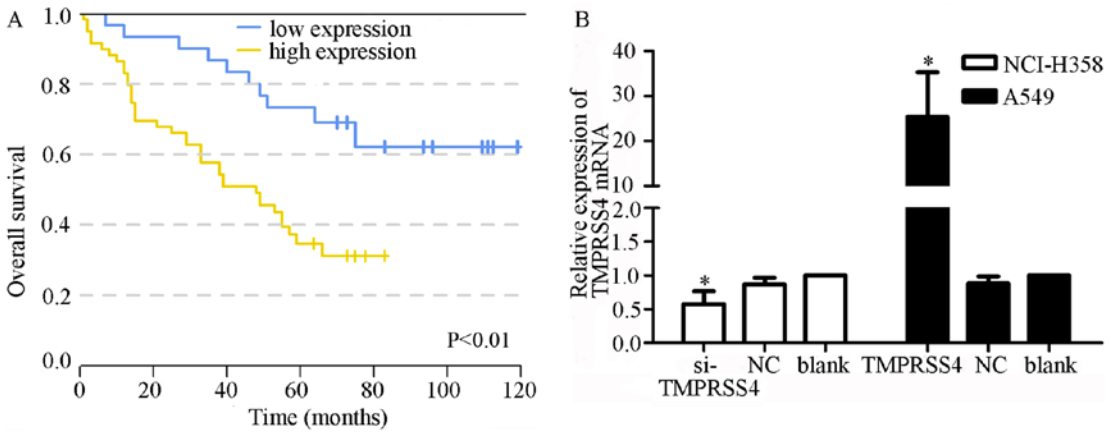


Figure 3. (A) Kaplan-Meier analysis of overall survival (months) in 89 patients with lung adenocarcinoma with a different expression of TMPRSS4 (log-rank test; P<0.01). The patients with a high expression had a poor prognosis compared with the patients with a low expression. (B) The relative mRNA expression of TMPRSS4 was elevated in the TMPRSS4 overexpression group and decreased in the si-TMPRSS4 group compared with the control groups; *P<0.05.

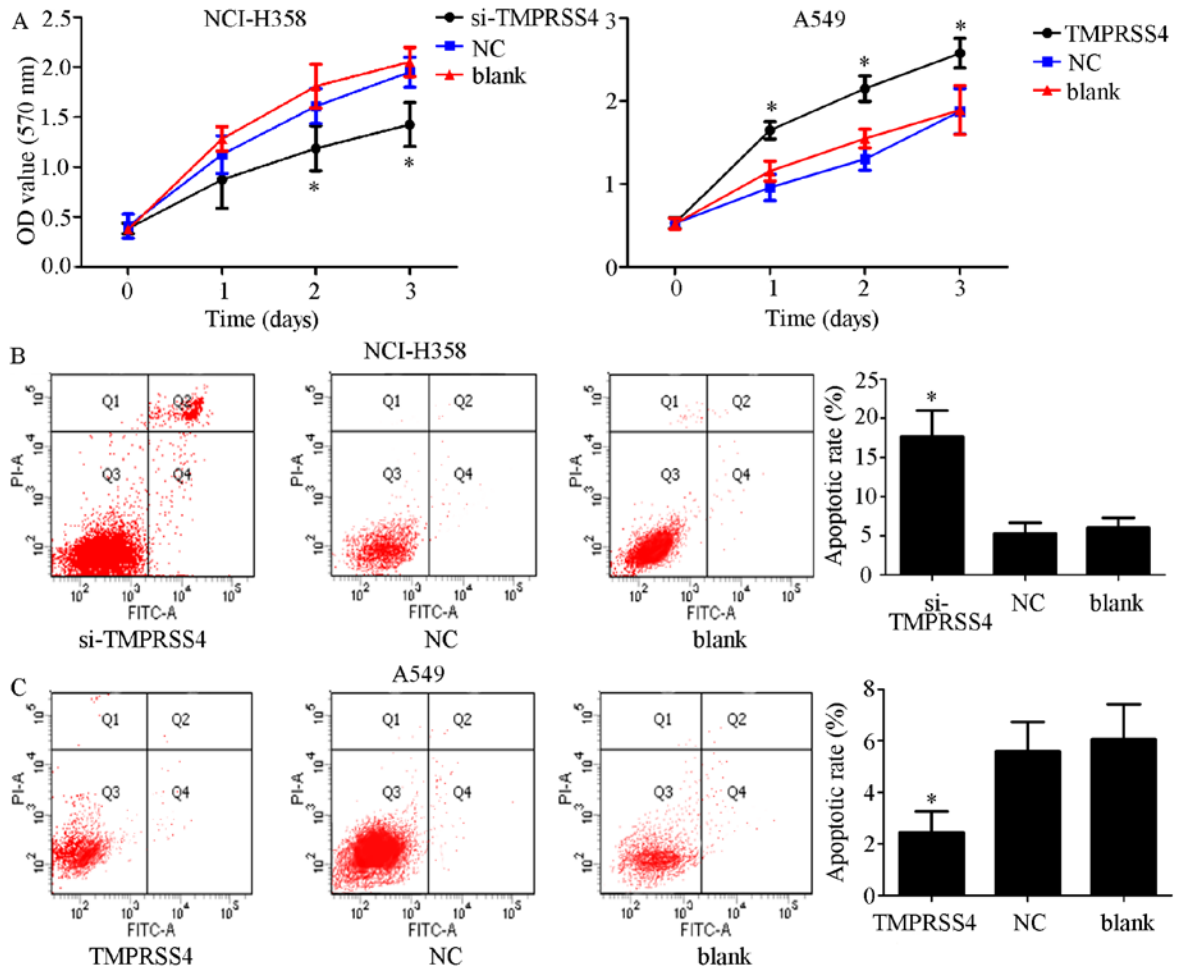


Figure 4. Effects of TMPRSS4 on lung adenocarcinoma (LUAD) cell lines. (A) Cell proliferation was detected by MTT assays following transfection of NCI-H358 cells with si-TMPRSS4, and transfection of A549 cells with TMPRSS4 overexpression vector. The results revealed that the silencing of TMPRSS4 inhibited cell proliferation and the enhanced expression of TMPRSS4 promoted cell growth compared with the control groups; *P<0.05. (B and C) Double staining with the Annexin V-FITC/PI kit was employed to measure apoptosis following transfection with si-TMPRSS4 or the TMPRSS4 overexpression vector into LUAD cell lines for 48 h. The results revealed that the apoptotic rate in the si-TMPRSS4 group was higher than that of the control groups; the overexpression of TMPRSS4 resulted in the marked reduction of cell apoptosis.

control groups; however, the apoptotic rate of the cells in the TMPRSS4 overexpression group was markedly lower (Fig. 4B and C, P<0.05), which suggested that TMPRSS4 may suppress cell apoptosis. These data suggested that a high expression of TMPRSS4 may enhance LUAD cell proliferation and inhibit cell apoptosis.

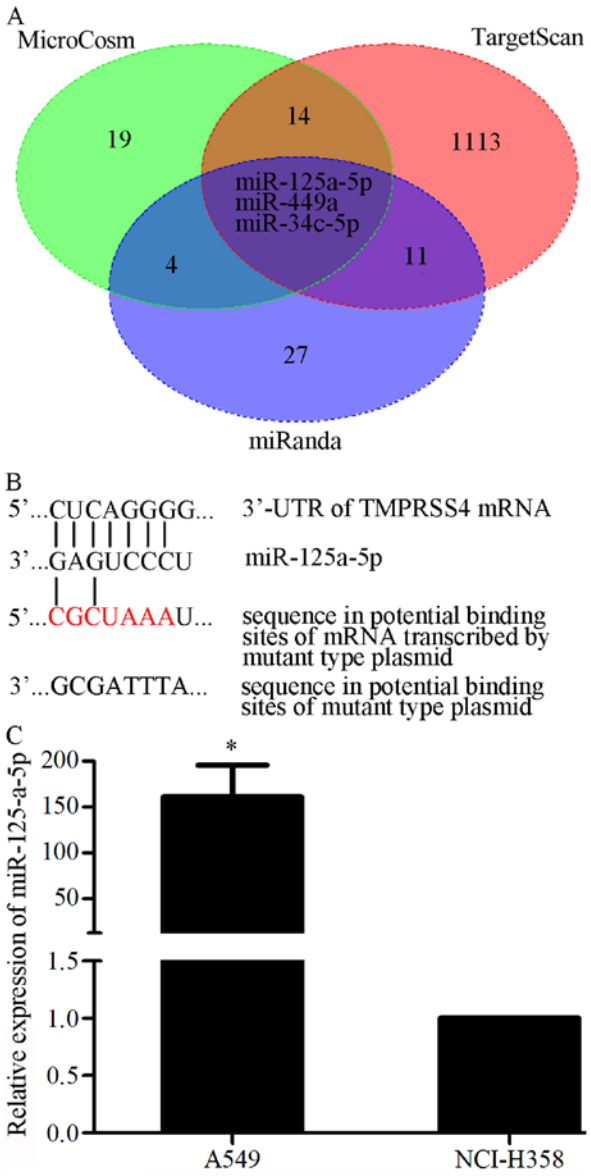


Figure 5. (A) Overlap analysis of MicroCosm, TargetScan and miRanda. miR-125a-5p, miR-34c-5p and miR-449a are most likely to act on the 3'-UTR of TMPRSS4 mRNA. (B) The potential binding sites of miR-125a-5p and TMPRSS4 mRNA, as well as the sequences in potential binding sites of mutant-type plasmid. (C) The relative expression of miR-125a-5p in A549 and NCI-H358 cell lines.

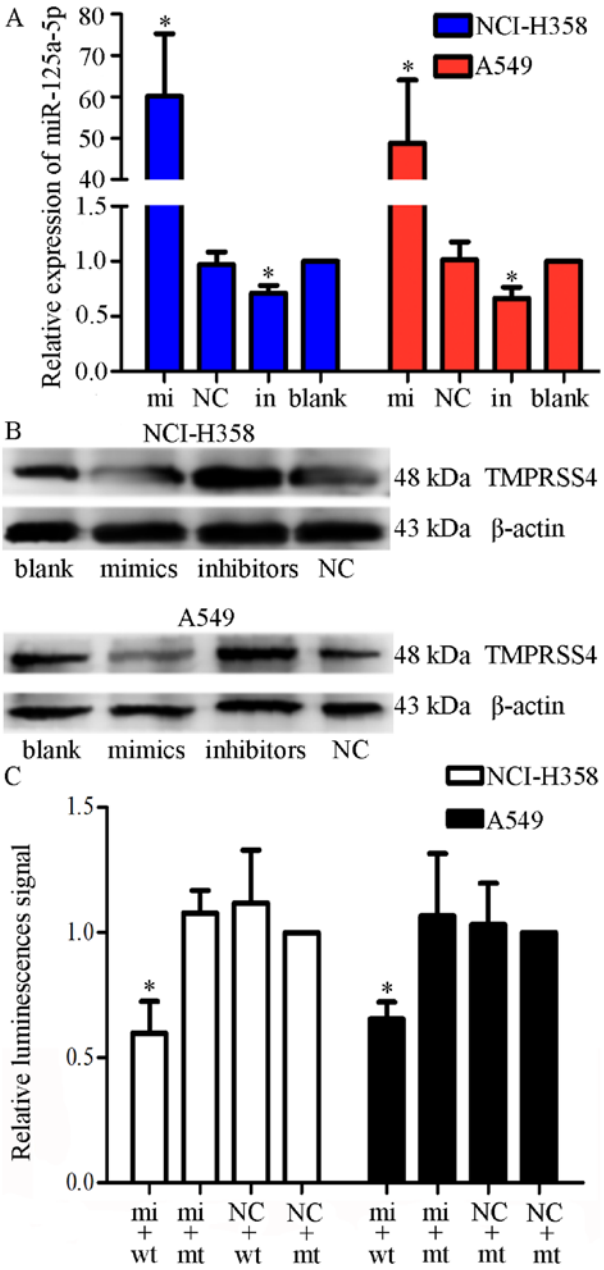


Figure 6. Direct regulation of TMPRSS4 by miR-125a-5p in lung adenocarcinoma (LUAD) cell lines. (A) The expression of miR-125a-5p was evaluated by RT-qPCR in LUAD cell lines following transfection for 48 h to confirm the transfection efficiency; *P<0.05. (B) Effects of miRNA mimics and inhibitors on the expression of TMPRSS4 in LUAD cell lines were assessed by western blot analysis 48 h following transfection. β-actin was used as a loading control. (C) Dual luciferase reporter assays were performed with vectors including the putative miR-125a-5p target sites in the 3'-UTR of TMPRSS4 mRNA (wild-type) and the control vector (mutant-type). Data were normalized by *Renilla*/firefly luciferase activity. In the miR-125a-5p + wild-type group, the relative luciferase activity was significantly lower, and no obvious change was observed in the other groups; *P<0.05. (The values for the mutant-type + NC group compared with pRL-TK vector were set equal to 1). Mi, mimics; NC, negative control of miRNAs; in, inhibitors; wt, wild-type; mt, mutant-type.

*Identification of potential miRNAs targeting TMPRSS4.* Three miRNA-target gene databases, miRanda, TargetScan (Release 6.2) and MicroCosm 5 were used to predict the miRNAs which suppress the expression of TMPRSS4. Following overlap analysis we acquired 3 potential miRNAs (Fig. 5A). Among these 3 miRNAs, miR-125a-5p was found to be downregulated in NSCLC and may have prognostic significance as previously reported by Zhu *et al* (13); thus, we decided to exmaine whether miR-125a-5p can inhibit the expression of TMPRSS4 via binding to the 3'-UTR of TMPRSS4 mRNA.

*TMPRSS4 is a direct target of miR-125a-5p.* The predicted binding sites of miR-125a-5p and TMPRSS4 mRNA, as well as the mutant sequences of the mt-TMPRSS4 plasmid are shown in Fig. 5B. To confirm the prediction, miR-125a-5p mimics and inhibitors, or NC-miRNA were transfected into the NCI-H358 or A549 cells. The transfection efficiency was confirmed by RT-qPCR (Fig. 6A). The results of western blot analysis following transfection indicated that the expression of TMPRSS4 was suppressed by the enforced expression

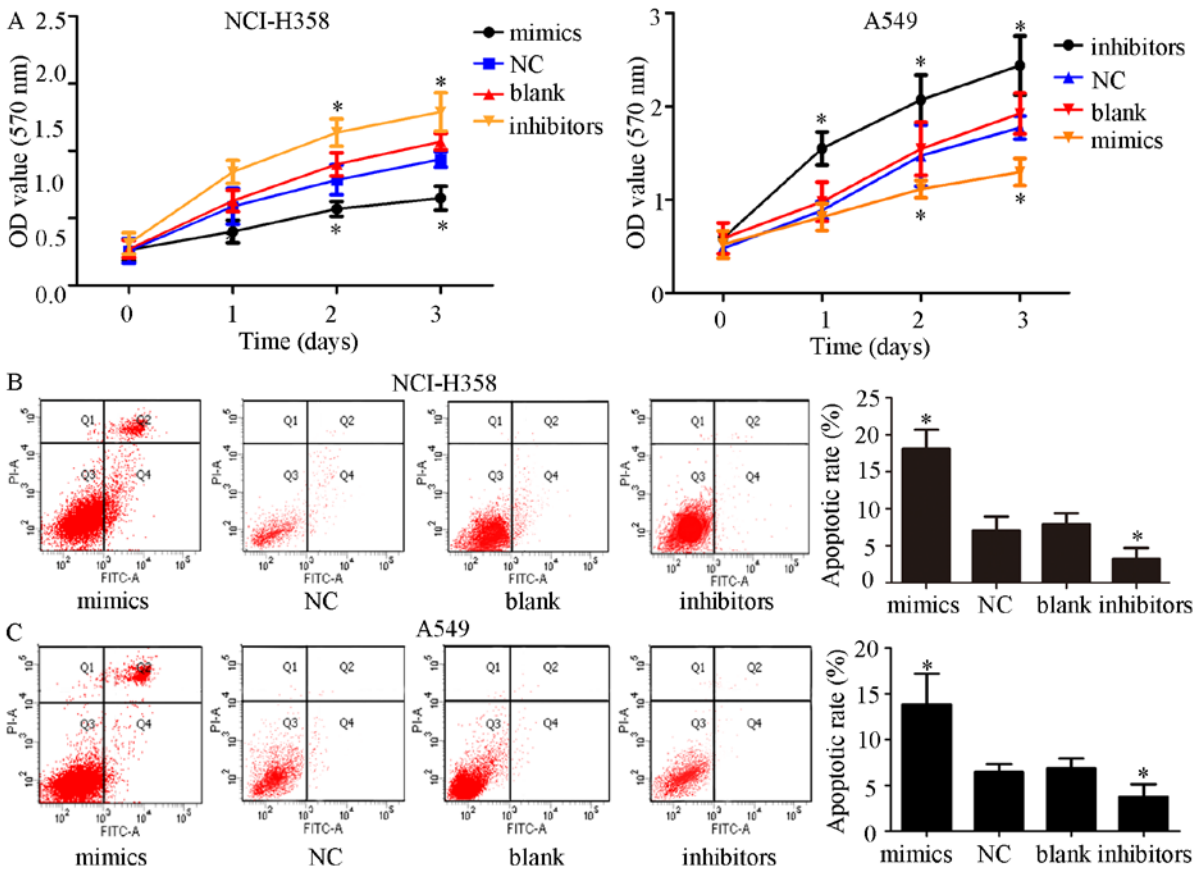


Figure 7. Effects of miR-125a-5p on lung adenocarcinoma (LUAD) cell lines. (A) Cell proliferation was determined by MTT assays following transfection with miR-125a-5p or its inhibitors. The results revealed that miR-125a-5p inhibited cell growth and the inhibitors promoted cell proliferation compared with the control groups; *P<0.05. (B and C) Apoptosis assays with Annexin V-FITC/PI kits were performed following miR-125a-5p or inhibitor transfection for 48 h. The results implied that the apoptotic ratio in the miR-125-5p group was higher than that of the control groups, and opposite effects were observed with the inhibition of miR-125a-5p.

of miR-125a-5p and was enhanced by the transfection with miR-125a-5p inhibitor, implying that miR-125a-5p may be a particular miRNA targeting TMPRSS4 in LUAD cell lines (Fig. 6B). Furthermore, dual luciferase reporter assays were performed to detect whether miR-125a-5p directly interacts with the 3'-UTR of TMPRSS4 mRNA, and either pGLO-wt-TMPRSS4 or pGLO-mut-TMPRSS4 with pRL-TK were transfected into NCI-H358 and A549 cells with miR-125a-5p or NC together. A marked decrease in the relative luciferase activity was observed in the miR-125a-5p + pGLO-wt-TMPRSS4 group compared with the other groups (P<0.05; Fig. 6C). Therefore, our results indicated that miR-125a-5p binds directly to the 3'-UTR of TMPRSS4 mRNA and negatively regulates TMPRSS4 expression in NCI-H358 and A549 cells.

*Effects of miR-125a-5p on NCI-H358 and A549 cells*. To examine the role of miR-125a-5p in LUAD cells, miR-125a-5p, inhibitor and NC were transfected, respectively into the cells in 6-well plates and MTT assays were utilized to measure cell proliferation. The results demonstrated that the ectopic expression of miR-125a-5p significantly inhibited cell proliferation, and miR-125a-5p suppression dramatically promoted cell growth compared with the control groups (Fig. 7A, P<0.05). Furthermore, cell apoptosis assays were conducted with the transfected NCI-H358 and A549 cells. The results indicated that the number of apoptotic cells was markedly higher in the miR-125a-5p group than in the control groups; however, the level of cell apoptosis was decreased in the cells in the inhibitor group (Fig. 7B and C, P<0.05), which implied that miR-125a-5p may be able to promote cell apoptosis. These data suggested that the effect of miR-125a-5p overexpression was consistent with TMPRSS4 neutralization, which attenuates proliferation and induces apoptosis.

*Overexpression of miR-125a-5p or knockdown of TMPRSS4 significantly inhibits the activation of the NF-κB signaling pathway*. To further investigate the regulatory mechanisms of miR-125a-5p and TMPRSS4 in LUAD cells, we focused on the NF-κB signaling pathway, which is involved in the action of TMPRSS4 on gastric cancer (21). Following transfection of miR-125a-5p mimic or si-TMPRSS4 into the NCI-H358 cells and the control groups, the expression of key proteins of the NF-κB signaling pathway was determined by western blot analysis. With miR-125a-5p enhancement or TMPRSS4 silencing, a markedly higher expression of IκBα was observed compared to the control groups, along with a decreased level of p-IκB expression. In addition, compared to the control groups, the expression of cytoplasmic NF-κB was elevated in the miR-125a-5p group and si-TMPRSS4 group, which reduces the expression of nuclear NF-κB. Furthermore, the expression level of Bcl-2, a direct transcriptional effector of the NF-κB

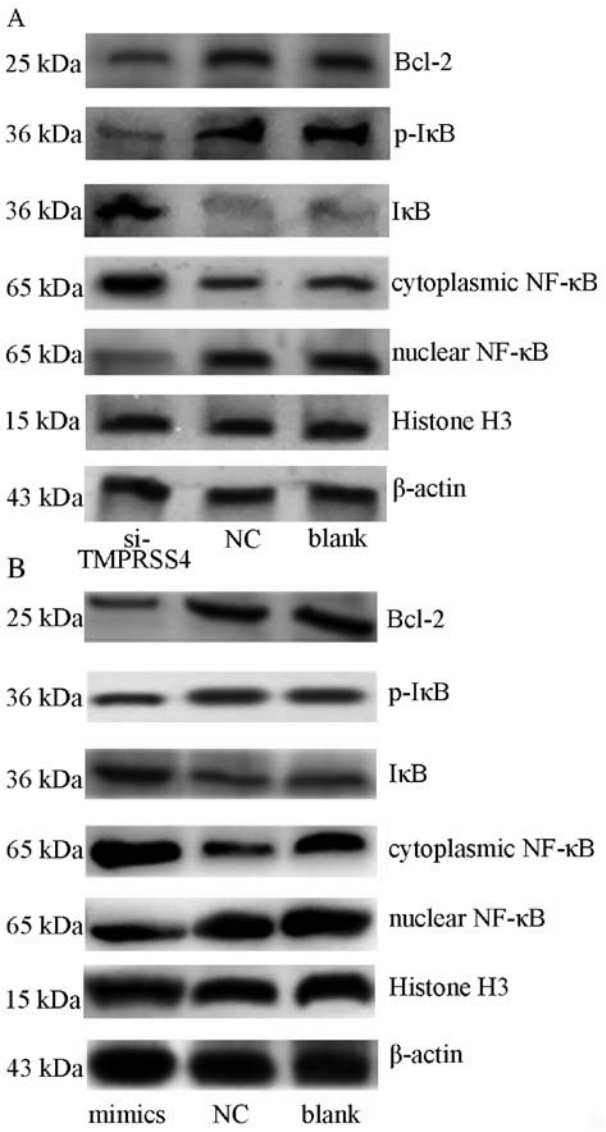


Figure 8. Silencing of TMPRSS4 or overexpression of miR-125a-5p inhibits the NF-κB signaling pathway in NCI-H358 cells. The phosphorylation of IκB was decreased with an increase in IκB expression in the si-TMPRSS4 group and mimics group, and cytoplasmic NF-κB expression was increased along with a decrease in nuclear NF-κB expression. These data indicated that the silencing of TMPRSS4 or the upregulation of miR-125a-5p may inhibit the activation of the NF-κB signaling pathway. Moreover, the expression of Bcl-2, which is the direct transduction effector of NF-κB signaling pathway, was also inhibited by si-TMPRSS4 or the overexpression of miR-125a-5p.

signaling pathway, was downregulated in the miR-125a-5p group and si-TMPRSS4 group compared to the control groups, suggesting that NF-κB activity was inhibited by miR-125a-5p or by the silencing of TMPRSS4 (Fig. 8).

*Survival analysis using online databases*. Kaplan-Meier plotter database (http://kmplot.com/analysis/index.php?p=service&cancer=lung) is an online survival analysis software to assess the prognostic value of biomarkers using transcriptomic data in NSCLC. Using this, we found that the result of the survival analysis of 866 patients with LUAD suggested that the patients with a low expression of TMPRSS4 had better prognosis than those with a high expression, which is agreement with our results (Fig. 9).

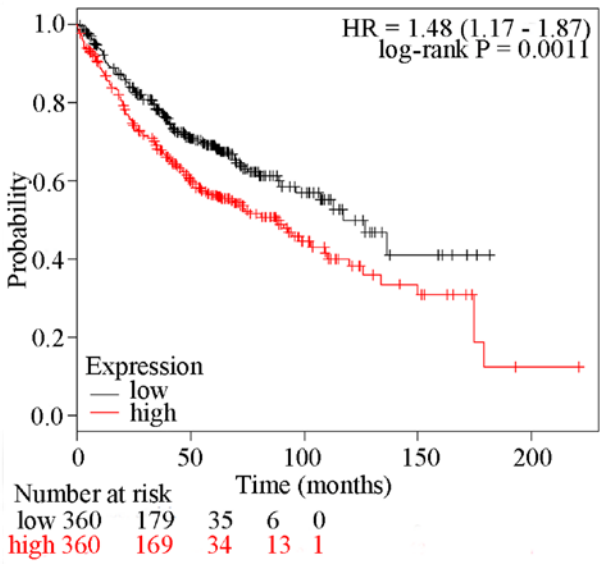


Figure 9. The results of survival analysis of 866 patients with LUAD from the Kaplan-Meier plotter database. The patients with a high expression of TMPRSS4 had a poor prognosis compared with those with a low expression; P<0.05.

## Discussion

TMPRSS4 is overexpressed in several solid tumors and may be assocaited with adverse outcomes in some of these tumors, such as breast cancer (22), NSCLC (17) and colorectal cancer (23). Moreover, TMPRSS4 is also capable of regulating self-renewal ability (24), inducing epithelial-mesenchymal transition (25,26), promoting cell proliferation (27) and invasiveness (21). TMPRSS4 in NSCLC has become a popular research topic in recent years. It has been reported that TMPRSS4 is an independent prognostic predictor of a decreased survival rate of patients with lung squamous carcinoma (6,7). Chikaishi *et al* demonstrated that TMPRSS4 expression was a marker of recurrence in patients with lung cancer (28). Moreover, TMPRSS4 can induce cancer stem cell-like properties in lung cancer cells and correlates with ALDH expression in patients with NSCLC (17,29). In the present study, we examined the expression of TMPRSS4 in tissues of both human LUAD and matched normal lungs by IHC. Of the tissues examined, the significant upregulation of TMPRSS4 was observed in the cancer tissues compared with the normal ones, which is similar to the findings of the study by Villalba *et al* (7). However, the results of the present study revealed that TMPRSS4 expression was associated with prognosis in 89 LUAD samples through Kaplan-Meier curve and Cox regression analysis; this finding differs from that in the study by Villalba *et al* (7). In our opinion, the reason why this phenomenon occurs is that we expanded the sample size. Likewise, the results of the analysis of the survival of 866 patients with LUADs from the Kaplan-Meier plotter database was in agreement with our findings. From this viewpoint, TMPRSS4 may be highlighted for use as a target for the treatment of LUAD or may have prognostic implications. In 2014, Larzabal *et al* pointed out that TMPRSS4 metastasis in NSCLC by regulating integrin α5 and miR-205 activity (30). Of note, the results of this study indicated that a higher TMPRSS4 expres-

sion was associated with a more advanced AJCC clinical stage, T stage and pathological grade, but not with the N or M stage. In our view, this may be due to the fact that most of our samples were selected from patients receiving surgery without neoadjuvant therapy, which excluded patients with metastasis and following potential bias. Nevertheless, 89 tissues was still a small number with which to draw a definitive conclusion; thus, we aim to continue our research and to include a greater number of patients with LUAD in future studies.

The positive correlation between the expression of TMPRSS4 and the T stage implies that TMPRSS4 may play a role in the growth of LUAD, as the latter is mainly affected by proliferation and apoptosis. To date, little is known about the association between TMPRSS4 and LUAD cell proliferation. Furthermore, apart from the study by Nguyen *et al* which demonstrated that TMPRSS4 was modulated by hypoxia (20), an in-depth exploration of the mechanisms underlying the regulation of TMPRSS4 in LUAD is warranted.

We utilized online databases for the identification of miRNAs involved in the regulation of TMPRSS4. The results suggested that miR-125a-5p may be a miRNA which is able to inhibit the transcription of TMPRSS4. Subsequently, RT-qPCR, western blot analysis and dual luciferase assay were employed to confirm the prediction. In reality, miR-125a-5p has been revealed to be closely associated with several types of cancer and may be a prognostic indicator in breast cancer (31), gastric cancer (32) and NSCLC (13). There are many genes identified as the targets of miR-125a-5p, such as ABL2 (33) and NAIF1 (11). Our study demonstrated that TMPRSS4 was a novel target of miR-125a-5p and that the decreased expression of miR-125a-5p may account for the increased expression of TMPRSS4 in LUAD. Moreover, the ectopic expression of miR-125a-5p may contribute to the induction of apoptosis and the suppression of cell proliferation, which was similar to the effect of the silencing of TMPRSS4. To investigate the underlying mechanisms, we examined the NF-κB signaling pathway, which is not only involved in migration and invasiveness, but also plays a role in proliferation and apoptosis (34,35). Of note, our results indicated that the activation of the NF-κB signaling pathway signaling pathway was significantly inhibited by the enhanced expression of miR-125a-5p or the silencing of TMPRSS4. To the best of our knowledge, this is the first study to report the ability of TMPRSS4 to partially affect LUAD cells.

Therefore, it can be concluded that an elevated level of TMPRSS4 is associated with the growth of LUAD via the NF-κB signaling pathway, and a low expression of miR-125a-5p, if not all, at least partially accounts for its overexpression. Thus, TMPRSS4 may be a potential candidate for a prognostic indicator, and therapies targeting the miR-125a-5p/TMPRSS4/NF-κB axis may have a bright prospect in the treatment of LUAD.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

**Authors' contributions**

XF made substantial contributions to the conception and design of the study; YiL was involved in data analysis; YaL was involved in the acquisition of the data for the study; YB was involved in the interpretation of the data for the study; XF, YiL, YaL and YB were involved in the drafting of the manuscript. CY made substantial contributions to the conception of the study and SX made substantial contributions to the design of the study; both CY and SX revised the study critically for important intellectual content. All authors gave the final approval of the final version of the study to be published and agree to be accountable for all aspects of the study in ensuring that questions related to the accuracy or integrity of any part of the study are appropriately investigated and resolved.

**Ethics approval and consent to participate**

For the use of patient samples, all the patients signed informed consent forms for the use of their samples in scientific research. The experiments were approved by the Ethics Committee of the First Hospital of China Medical University (IRB Approval 2012-40-2).

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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