RBM10, a new potential RBP function, recruits METTL3 to induce N6-methyladenosine-MALAT1-dependent modification on inhibiting the invasion and migration of NSCLC

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Abstract

Background: Previous studies have shown that RBM10 is a potential tumor suppressor protein that can inhibit proliferation and promote apoptosis of NSCLC. RBM10 can bind and modify RNA at the post transcriptional level.

Method and results: Transwell and cell wound healing assay showed that RBM10 significantly inhibited the invasion and migration of NSCLC. CLIP-seq showed that among all RBM10 binding RNAs, lncRNA MALAT1 with high m6A methylation in lung cancer had the highest binding peak among all non-coding RNAs. RNA Immunoprecipitation has verified the direct combination of RBM10 and MALAT1. The rescue experiment confirmed that RBM10 affected the phosphorylation of PI3K/AKT/mTOR passway protein, the invasion and migration ability by regulating MALAT1. MeRIP-qPCR confirmed that RBM10 could inhibit MALAT1 m6A methylation levels by recruiting METTL3.

Conclusion: These results suggest that RBM10, as an RNA-binding protein, may inhibit m6A methylation of MALAT1 by recruiting METTL3, and affect phosphorylation of the downstream PI3K/AKT/mTOR pathway by binding and regulating MALAT1, ultimately affecting the invasion and migration of NSCLC.

1. Introduction

Post transcriptional regulation of RNA is a process tightly controlled by regulatory factors, of which RNA binding protein (RBP) is the most important regulatory factor.[1] By binding with RNA, they participate in the processes of RNA splicing, transport, stability and degradation, and affect important physiological processes such as apoptosis, proliferation and differentiation.[2] RNA binding motif protein 10 (RBM10) is one of the most important members of RBP family. The abundance and activity can affect the splicing pattern of tumor related genes, thus affecting the occurrence and progression of tumors. The inhibitory effect of RBM10 on lung cancer, breast cancer, liver cancer, pancreatic cancer and other malignant tumors has been confirmed: RBM10 can promote tumor apoptosis, inhibit invasion, metastasis and cell proliferation. [3, 4]

Metastasis associated lung cancer transcript 1 (MALAT1) is an important member of the long non coding RNA (lncRNA) family. Studies have found that the expression level of MALAT1 increases in a variety of tumors through endogenous competitive RNA (ceRNA) mechanism activates the classical pathway pI3K/AKT/mTOR, plays a role in promoting tumor invasion and metastasis. [5, 6] In non-small cell lung cancer (NSCLC), MALAT1 is also highly expressed and is closely related to the poor prognosis of patients. [7] Therefore, blocking and targeting MALAT1 may become an important link in the targeted treatment of NSCLC. PI3K/AKT/mTOR signaling pathway has been used as a potential therapeutic target in the treatment of a variety of tumors. Inhibitors of this signaling pathway have been proved to have obvious antitumor effects in vitro and in vivo experiments.[8] The preliminary results of our research group also confirmed that RBM10 can affect the expression of AKT protein. Whether the expression of RBM10 has an impact on MALAT1 and PI3K/AKT/mTOR pathways is one of the direction of our research.

N6-methyladenosine methylation (m6A), the most common (more than 60%) RNA epigenetic modification, can add a methyl group to the N atom at position 6 of mRNA and lncRNA adenine.[9] Studies have proved that the abnormal regulation mechanism can induce the abnormal expression of downstream target genes and eventually lead to tumor.[10] M6A methylation is composed of methyltransferases (writers), demethylases (erasers) and m6A binding proteins (readers).[11] Once these enzymes are abnormal, they will cause a series of diseases, including cancer, neurological diseases and embryonic retardation. Recent studies have shown that there is an m6A "switch" in MALAT1 structure, which is catalyzed by m6A methyltransferases (writers). When it turned on, MALAT1 undergoes m6A methylation, resulting in changes in its hairpin structure. This structural change can affect the mechanism of MALAT1 binding to protein.[12] It can control the mature translation and decline of other downstream mRNAs.[13] As the initiating factor of this mechanism, the study on the structure and function of m6A methyltransferase complex is very important. It has been reported that RNA binding motif 15 (RBM15), as a member of RNA binding motif protein family, can interact with m6A methyltransferase complex METTL3/METTL14/WTAP (methyltransferase like 3/ methyltransferase like 14/WTAP) and adjust the dynamic level of m6A. Therefore, whether RBM10, which belongs to the RNA binding motif protein family with RBM15, can regulate and how to regulate m6A methylation of MALAT1 is the focus of our research.

2. Materials And Methods

2.1 immunohistochemistry

For immunohistochemistry (IHC) analysis, paraffin embedded tissues were dewaxed and antigenic repaired at 115 ℃ for 20 minutes using a Tris-EDTA (PH = 9.0) based unmasking solution. After the endogenous peroxidase activity was blocked with 3% hydrogen peroxide, the nonspecific antigen was blocked with 10% goat serum and cultured at room temperature for 30 minutes. Then the primary antibody was cultured overnight at 4℃. Wash with PBS three times for five minutes each time. The sections were stained with 3 '- diaminobenzidine (DAB) with tissue stain streptavidin peroxidase Kit (sp9001, zszb bio, Beijing, China), and counterstained with hematoxylin. All tissue sections were photographed using dp72 microscope (Olympus, Tokyo, Japan). The immunoreactivity was qualitatively evaluated by percentage of positive staining cells.

2.2 Western blot and immunoprecipitation

After different treatments, cells or tissues were lysed and separated in lysis buffer. The supernatant was collected after centrifugation. The protein concentration was determined by BCA kit. (Beyotime, Jiangsu, China) The protein was separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. PVDF membrane was sealed in 5% skimmed milk and incubated at room temperature for 1.5 hours, and then co incubated with specific primary antibody at 4 ℃ overnight. The next day, the membrane was cleaned with TBST for 3 times, each time for 10 minutes, and then further combined with horseradish peroxidase to bind secondary antibody for 1 hour at room temperature. After washing the

membrane three times, each time for 10 minutes. The bands were detected by Amersham ECL Plus Western Blotting Detection Reagent (Millipore) and quantified using image J. The repeatability of the experiment is: by repeating at least three times.

Antibodies: anti-GAPDH (Proteintech, 10494-1-AP,1:1000), anti-RBM10(Abcam, ab72423,1:1000), anti-AKT(CST, 9272, 1:1000), anti-pAKT (CST, 4060, 1:1000), anti-PI3K(CST, 4292, 1:500), anti-pPI3K(CST,4228,1:500), anti-mTOR(CST, 2972, 1:1000), anti-pmTOR (CST, 2974, 1:1000), anti-E cadherin(Abcam, ab212059, 1:1000), anti-vimentin(Abcam, ab20346, 1:1000), anti-rabbit m6A(Synaptic system, GER), anti-METTL3(Proteintech, 15073-1-AP, 1:500), anti-rabbit IgG(Abcam, ab172730, 1:1000)

2.3 RNA isolation, qRT-PCR

The samples were transferred to ribonuclease/ deoxyribonuclease free tubes and Store at -80℃ for total RNA extraction. Total RNA was extracted with T-Trizol LS reagent (Invitrogen, Carlsbad, CA, USA). For qRT-PCR analysis, total RNA from plasma was firstly reverse transcribed into complementary DNA using PrimeScript™ RT reagent kit with gDNA eraser (Takara, Dalian, China) as follows: 37 ℃ for 15 minutes, then 85 ℃ for 15 minutes 5 seconds. Then, real-time PCR was performed using SYBR premix Ex Taq (Takara). Thermal cycle conditions are as follows: 95 ℃ for 30 seconds, followed by 95 ℃ 40 cycles for 5 seconds, 60 ℃ for 30 seconds, 72 ℃ for 30 seconds, followed by a final cycle of 2 minutes at 72 ℃. QRT-PCR results were calculated using the 2^−ΔΔT method. β-actin expression was used for normalization.

MALAT1 forword: 5' AAGTTAAATATGAGCCACTG 3' MALAT1 reverse: 5' CACTACCATATCCAAACAAC 3'

β-Actin forword: 5' GTGGCCGAGGACTTTGATTG 3'

β-Actin reverse: 5' CCTGTAACAACGCATCTCATATT 3'

2.4 Cell Culture and Reagents

Non-small cell lung cancer cell lines A549 and H460 were obtained from the Chinese Academy of Medical Sciences (Beijing, China) and cultured in Dulbecco's modified Eagle's medium or RPMI-1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum at

37℃ in a humidified atmosphere containing 5% CO_2. Mycoplasma contamination tests were performed and a brief validation was performed Tandem repeat (STR) polymorphism analysis was performed by Genetica DNA lab.

2.5 Specimen and tissue chips

All lung cancer tissue specimens (51 cases) were collected from the ethics Committee of the Hospital and Research Institute of the Second Medical College of Jilin University. The study was approved with the prior written consent of all patients. All patients were diagnosed according to the World Health Organization's lung cancer standard of tumor (T), lymph node (N), metastasis (M) staging system of lung cancer.

2.6 Vector construction and cell transfection

The lentivirus gene vector was constructed from Jikai Gene (shanghai, China). Lipo2000 was added to make the vector enter 293T cells. The lentivirus was harvested 48 hours after transfection and stored in the refrigerator at

− 80 ℃. Then A549 and H460 cell lines were transfected into six-well plates when the cell density reached 70%. Using low serum medium and adding lentivirus vector of target gene. They could be divided into negtive control group, (NC-oe and NC-sh), RBM10 overexpression group (oe-RBM10) and RBM10 RNA interference (sh-RBM10) group. The next day, it was changed to 10% complete serum medium. Puromycin was used to screen the transfected cell lines.

2.7 Transwell assay

Transfer cells 1×10^5 placed on a 24 well plate covered with 8% matrix adhesive (Thremo,#142475) in the upper compartment, which was put into serum-free medium. The complete medium containing 10% serum was placed in the lower compartment. The system cultured for 24 hours. The chamber was fixed with 4% paraformaldehyde for 15 minutes, and stained with 0.5% crystal violet for 15 minutes. After the non-invasive cells were removed, the invasive cells were counted under the microscope, and 5 non replication regions were randomly captured and counted Cells.

2.8 Wound-healing assay

The cells were grown in serum-free modified medium at 37 ℃ and 5% CO_2. After adherence, 10% fetal bovine serum was supplemented the next day. The adherent cells were cut with the tip of 200 μl pipette and cultured for 36h. The average degree of wound closure was quantified and the percentage of wound healing was calculated.

2.9 Immunofluorescence

Transfected A549 and H460 cells were inoculated in confocal dishes (4×10^4 cells / well). After cell adhesion, the cover glass was fixed with 4% paraformaldehyde at room temperature for 30 minutes, and then fixed with 0.5% Triton-x extract 100 solution for 10 minutes. Then, 5% bovine serum albumin was placed at room temperature for 30 minutes, and the primary antibody solution was cultured at 4 ℃ overnight. After washing with PBS, Goat anti rabbit IgG (H + L) with fluorescence group (Wuhan Proteintech, China) was further purified at room temperature for 1 hour, and soaked with DAPI (Solarbio, Beijing, China) for 10 minutes at room temperature. Anti-fluorescence quencher was added and observed under fluorescence microscope. (Olympus, Japan) Different microscope images were randomly selected for qualitative analysis.

2.10 Crosslinking-immunprecipitation and high-throughput sequencing (CLIP-seq)

RNA molecules and RNA binding proteins are coupled under UV irradiation. After the RNA protein complex is precipitated with the specific antibody of RNA binding protein, the RNA fragments are recovered, and these molecules are sequenced by adding connectors, qRT-PCR and other steps. The data was download from GEO (*ncbi.nlm.nih.gov/geo*) database. (Appreaciate the beauty of life, Wuhan)

2.11 RNA Binding Protein Immunoprecipitation (RIP)

Frozen bacteria was treated with lysozyme, then lysed in ice-cold lysis buffer with freshly added 1mM DTT, 200 U/mL RNase inhibitor (Takara) and protease inhibitor cocktail (Roche) for 30 min once. After centrifugation for 30 minutes at 12000 rpm at 4℃, the supernatant was transferred to a new tube and used for immunoprecipitation. For immunoprecipitation, 500 μL lysate was incubated with 10 μg anti-flag antibody (Sigma) or control IgG antibody overnight at 4℃. The immunuprecipitates were further incubated with protein A dynabeads for 1h at 4℃. After applying to magnet and removing the supernatants, the beads were sequentially washed with lysis buffer, high-salt buffer for two times, respectively. The immunoprecipitates were eluted from the beads with elution buffer and the RNA was purified with trizol reagent (Life technologies). Purified RNAs were thermal treatment at 65℃ 5min, followed by reverse transcription was performed with RT primer harboring 3' adaptor sequence and randomized hexamer. The cDNAs were amplified by 2X Dream Taq Mix(Thermo), PCR products electrophoresis analysis by agarose gel .

MALAT1 forword primer:

5' AAGTTAAATATGAGCCACTG 3'

MALAT1 reverse primer:

5' CACTACCATATCCAAACAAC 3'

2.12 SiRNA design and transfection

Sh-RBM10 cell lines were transfected with siRNA using X-treme GENE™ siRNA Transfection Reagent. 6-8h after lipofection, the cell was substituted with fresh complete mediums. After another 24h, protein expression and cell viability were tested by western blotting and qRT-PCR analysis, respectively.

Si-MALAT1-homo-7975 sense:

5' CCAUUAAUUUGCCUGCAAATT 3'

Si-MALAT1-homo-7975 antisense:

5' UUUGCAGGCAAAUUAAUGGTT 3'

Si-MALAT1-homo-236 sense:

5' GUGGUGGUAUUUAGAUAAATT 3'

Si-MALAT1-homo-236 antisense:

5' UUUAUCUAAAUACCACCACTT 3'

Si-MALAT1-homo-4210 sense:

5' CCAGAGAACUUAAAGUCUUTT 3'

Si-MALAT1-homo-4210 antisense:

5' AAGACUUUAAGUUCUCUGGTT 3'

2.13 Methylated RNA Immunoprecipitation-qPCR (MeRIP-qPCR)

DNase I digested an appropriate amount of total RNA to obtain RNA fragments with a length range of 60-200BP. Dynabeads protein beads and anti m6A antibodies were pre-mixed and cultured at 4℃ for 2h to obtain the complex of magnetic beads and antibodies. The fragment RNA was added to the above magnetic bead antibody solution and cultured at 4℃ for 2 hours, separated the magnetic beads and solution with a magnetic frame, removed the solution, added washing buffer to resuspend the magnetic beads, washed them fully for 3–5 times, added lysate to the magnetic beads that removed nonspecific binding RNA, recovered the m6A modified fragment and labeled it as IP RNA. Using IP and 10% input RNA as template, the random primer binding in cDNA synthesis was completed in the buffer containing random primers, and a cDNA was obtained by reverse transcription reaction under the action of primescript RT enzyme as quantitative template; Fluorescence quantitative PCR was performed using m6A modified site-specific primers. For specific steps, see TB green ™ Premixed dimer ™ (perfect real time) kit description.

2.14 Statistical analysis

All data were reported as mean ± standard deviation (SD). Statistical analysis was performed using graphpad prism 8.0 software (GraphPad Software, Inc. La Jolla, CA). Data were analyzed for significance using Student's t-test (two groups) or one-way ANOVA with Tukey's post hoc tests (multiple groups). A value of $P < 0.05$ was considered statistically significant. Pearson's Chi-squared test was performed to determine the association of RBM10 with clinicopathological characteristics.

3. Results

3.1 RBM10 expression is lower in NSCLC tissue than in para-cancerous tissue

We measured RBM10 protein expression in 51 pairs of cancer and para-cancer tissue samples. There were 26 of the specimens from patients with lung adenocarcinoma (LUAD) and the rest were from patients with lung squamous cell carcinoma (LUSC). Western blotting result showed that RBM10 expression was lower in LUAD and LUSC than in para-cancer tissues. (Fig. 1B) We also examined the expression of RBM10 in NSCLC and normal tissues by immunohistochemistry. (Fig. 1A) The results were consistent with western blot. This further confirms our view that RBM10 is a tumor suppressor.

3*.2 MALAT1 content in non-small cell lung cancer tissues was higher than that in adjacent tissues.*

QRT-PCR was used to detect MALAT1 expression in LUAD tissues, LUSC tissues and adjacent tissues. The results showed that MALAT1 levels were higher in cancer tissues than in adjacent tissues. (Fig. 1C)

3.3 Clinical correlation analysis of RBM10 and MALAT1

We analyzed the effects of RBM10 and MALAT1 expression on cancer survival by GEPIA (http://gepia.cancer-pku.cn/*).* The results showed that overall survival and disease-free survival were longer with high expression of RBM10 than with low expression of RBM10. The expression of MALAT1 had little effect on overall survival, but the long-term survival rate (> 150 months) of low expression was higher than that of high expression. Disease-free survival of high MALAT1 expression state was higher than low expression state. (Fig. 1D) RBM10 protein levels were associated with gender, but not with histological types, age, the T and N stages of advanced tumors, or smoking status. (Table 1.)

3.4 RBM10 inhibits the invasion and migration of NSCLC cells

We first performed a functional in vitro analysis to explore the role of RBM10 in the progression of non-small cell lung cancer. RBM10 over expression or silencing was performed in A549 and H460 cells by lentivirus transfection, for details of grouping for *methods 2.6*. The expression efficiency was measured by western blotting (Fig. 2A). The results of transwell experiment and wound healing experiment showed that the overexpression of RBM10 significantly reduced the ability of cell invasion and migration, and RBM10 silencing significantly enhanced the ability of cell invasion and migration respectively. (Fig. 2B and Fig. 2C) As we know, EMT is the key process of cell invasion and migration. According to the results of immunofluorescence qualitative analysis and western blotting quantitative analysis, RBM10 can affect the expression of EMT related proteins E-cadherin and vimentin in A549 and H460 cell lines. (Fig. 3A and Fig. 3B) Specifically, RBM10 can increase the expression of E-cadherin and reduce the expression of vimentin. On the contrary, silencing RBM10 can inhibit E-cadherin and increase the expression of vimentin.

Table 1
Association of RBM10 with clinicopathological characteristics from 51 NSCLC patients

characteristic	N (%)	RBM10 expression N(%)		P value
		High expression	Low expression	
Histological types				
Squamous cell	25(49.02)	13(52.00)	12(48.00)	0.4043
adenocarcinoma	26(50.98)	10(38.46)	16(61.54)	
Age(years)				
≤60	28(54.90)	10(35.71) 1010(35.71)	18(64.29)	0.7737
>60	23(45.10)	10(43.48)	13(56.52)	
gender				
male	32(62.75)	7(21.87)	25(78.13)	**0.0151**
female	19(37.25)	11(57.89)	8(42.11)	
smoke				
No	30(58.82)	14(46.67)	16(53.33)	0.7793
Yes	21(41.18)	11(52.38)	10(47.62)	
PT status				
T1-T2	28(54.90)	10(35.71)	18(64.29)	> 0.9999
T3-T4	23(45.10)	8(34.78)	15(65.22)	
PN status				
PN-	27(52.94)	10(37.04)	17(62.96)	> 0.9999
PN+	24(47.06)	8(33.33)	16(66.67)	

p-values represent Pearson χ^2 test; PT status, Tumor; PN status, Node. Bold values indicate $P < 0.05$.

3.5 RBM10 can inhibit the phosphorylation of PI3K/AKT/mTOR pathway.

PI3K/AKT/mTOR pathway is a cellular pathway closely related to RBM10 and the invasion and metastasis of NSCLC. According to our western blotting results, RBM10 can affect the phosphorylation of this pathway. Specifically, RBM10 inhibit the expression of p-PI3K, p-AKT and p-mTOR, while RBM10

silencing promote the phosphorylation expression of the above proteins. (Fig. 3C) The total protein expression of PI3K, AKT and mTOR was not affected.

3.6 RBM10 directly binds and regulates the expression level of MALAT1

In order to study which lncRNA RBM10 will affect, we used CLIP-seq technology. CLIP-seq is a revolutionary technology to reveal the interaction between RNA molecules and RNA binding proteins at the genome-wide level. According to CLIP-seq results, RBM10, as an RNA binding protein, bind 4040 RNAs in both data repetitions at the genome wide level, including 357 lncRNAs among all. MALAT1 is the highest in terms of binding tags, and MALAT1 ranks third in terms of binding max-height. (Fig. 4A) The above evidence fully suggests that RBM10 may be associated with MALAT1. In order to continue the verification at the molecular level, we used RIP technology to confirm that RBM10 protein does have a direct binding relationship with MALAT1. It is a technology to study the binding of RNA and protein in cells, and it is a powerful tool to understand the dynamic process of post transcriptional regulatory network. Compared with input group or IgG group, RBM10 IP precipitation group significantly bound MALAT1. (Fig. 4E) We have reason to doubt that RBM10 can regulate long-chain noncoding RNA MALAT1. Next, we performed qRT-PCR in A549 and H460 cell lines transfected with RBM10. The results showed that the level of MALAT1 decreased in RBM10 overexpressed cell lines and increased in RBM10 silenced cell lines. (Fig. 4B) The above results show that RBM10 is closely related to MALAT1. RBM10 can not only reverse regulate the expression of MALAT1, but also directly combine with MALAT1.

3.7 RBM10 regulates NSCLC invasion and migration and phosphorylation of PI3K/AKT/mTOR pathway through MALAT1.

The above results have confirmed that the level of MALAT1 is increased in RBM10 silenced group. We used siRNA technology to reduce the level of MALAT1 in this group. Among them, siRNA236 decreased the most. Therefore, in the three groups of siRNA (siRNA236, siRNA7975 and siRNA4210), we chose siRNA236 for subsequent experiments. (Fig. 5A) As confirmed by previous experimental results, the phosphorylation level of PI3K/AKT/mTOR pathway in sh-RBM10 group was still higher than that in negtive control, after inhibiting the level of MALAT1, the phosphorylation level of PI3K/AKT/mTOR pathway protein was decreased again. (Fig. 5B) Similarly, the rescue experiment is also meaningful in the phenotype of invasion and migration. Specifically, compared with negtive control, the degree of invasion and migration of sh-RBM10 group was increased. After inhibiting the level of MALAT1, the degree of increased invasion(Fig. 5C)and migration (Fig. 5D) fell back again. That means, RBM10 regulates NSCLC invasion and migration and phosphorylation of PI3K/AKT/mTOR pathway protein through MALAT1.

3.8 RBM10 can inhibit the m6A methylation level of MALAT1 by recruiting METTL3.

M6A modification site prediction is based on SRAMP online software (http://www.cuilab.cn/sramp*)* and some reported high-throughput sequencing data. It is mainly based on whether there is a typical m6A modified motif in the sequence. The reliability of possible methylation modification sites can be divided into four levels: very high confidence, high confidence, moderate confidence and low confidence. By default, only the sum of sites with the level of "very high confidence" is given: 2539, 2601, 2635,2649, 2656 and 2744. (Fig. 6A) A pair of primers shall be designed according to each m6A modification site for detection. The same pair of primers can be used for detection of m6A sites within 120nt. The number of primers shall be subject to the actual design. Therefore, we designed three primers and covered six methylation sites of MALAT1, which were divided into cluster1, cluster3 and site12. (Fig. 6A) The experimental results show that with input as the control, the m6A methylation degree of MALAT1 in each group was calculated (input%). The methylation degree of MALAT1 m6A level between NC-oe and oe-RBM10 groups, NC-sh and sh-RBM10 groups was analyzed respectively. The results show that in three group sites of MALAT1 (cluster1, cluster3 and site12), when RBM10 is overexpressed, the methylation level of MALAT1 decreases, and when RBM10 is silent, the methylation level of MALAT1 increases, indicating that RBM10 can inhibit the m6A methylation level of MALAT1. (Fig. 6B)

In order to further study the mechanism how RBM10 can affect the methylation level of MALAT1 m6A, we conducted a more in-depth study on the key enzymes of methylation modification of RBM10 and MALAT1 m6A. Previous studies have pointed out that METTL3 is a key methylase modified by MALAT1 m6A. Therefore, we conducted IP experiments on RBM10 and METTL3. The experimental results showed that there was a binding relationship between RBM10 and METTL3. (Fig. 6C) The interaction between METTL3 and RBM10 was analyzed by STRING protein interaction (PPI) database. This indicates that RBM10 is likely to regulate the methylation process of MALAT1 by recruiting METTL3. (Fig. 6D)

4. Disscusion

Post transcriptional regulation of RNA is a process tightly controlled by regulatory factors. RNA binding protein (RBP) is the most important regulatory factor. RBP is a kind of protein that can bind RNA. It participates in RNA splicing, transport, stability and degradation, and affects important physiological processes such as apoptosis, proliferation and differentiation. RNA binding motif protein 10 (RBM10) is one of the most important members of RBP family. At present, studies have shown that RBM10 can inhibit the occurrence and development of tumors, and its tumor suppressor effect in lung cancer, breast cancer, liver cancer, pancreatic cancer and other malignant tumors has been confirmed.

In order to clarify the role of RBM10 in non-small cell lung cancer, we evaluated the expression level of RBM10 in human NSCLC tissue samples. The results showed that the expression of RBM10 decreased in NSCLC tissues, and its content was lower than that in normal and adjacent tissues. The reason may be the mutation of RBM10 protein in patients, resulting in the decrease of expression, which can not effectively inhibit the biological process related to cancer cells, and then lead to a series of follow-up processes of cancer occurrence and development. Then we analyzed the correlation between the expression of RBM10 and clinical data, and found that the expression of RBM10 was related to gender. In the samples collected by this project, the expression of RBM10 in male patients was generally lower with

significant difference. Previous studies have pointed out that RBM10 is often accompanied by gene mutations in the form of point mutation, insertion mutation, missense mutation and nonsense mutation in male patients, [14, 15, 16] resulting in low expression rate, which is coupled with the trend of the research results of this project. We also found that patients with high expression of RBM10 had longer overall survival and disease-free survival, suggesting that RBM10 may have the potential as a prognostic index. The main reason for the shortened survival time of patients is tumor invasion, growth and distant metastasis. Patients with high expression of RBM10 can obtain a long survival time. Therefore, it is speculated that RBM10 may be involved in inhibiting the invasion and metastasis of lung cancer directly related to long-term survival. EMT is not only a biological process of epithelial cells transforming into stromal cells, but also a highly dynamic and reversible key step in the process of tumor cell invasion and migration. Its main features include the reduction of the expression of cell adhesion molecule (E-cadherin), the transformation of cytokeratin skeleton into vimentin dominated cytoskeleton, and so on. [17] The loss of E-cadherin expression is a landmark change in EMT occurrence, tumor invasion and migration,[18] which is usually accompanied by the up regulation of N-cadherin and vimentin expression. [19] We found that RBM10 could promote the expression of E-cadherin and inhibit the expression of vimentin, which proved that RBM10 was involved in delaying the EMT process of NSCLC cells. Further Transwell invasion experiment and scratch experiment confirmed that RBM10 could weaken the invasion and migration ability of NSCLC, that is, RBM10 could inhibit the EMT process and invasion and migration of NSCLC.

In order to explore the mechanism by which RBM10 inhibits the invasion and migration of NSCLC, we found that PI3K/AKT/mTOR pathway is the key signal pathway of tumor invasion and migration, which has been confirmed in a variety of tumors. In this study we confirm that RBM10 affects the activation of this pathway by down regulating the phosphorylation level of this pathway protein, and finally inhibits the invasion and migration of NSCLC.

In the whole genome, RNA encoding protein only accounts for 3% – 5%, and most of the rest are non coding RNA, of which lncRNA accounts for about 75% of the whole human genome sequence.[20] LncRNA can regulate gene expression at different levels such as transcription, splicing, methylation, mRNA stability and translation, and affect a variety of cell biological processes such as migration, proliferation, apoptosis and invasion. As an RNA binding protein, there are few reports on RBM10 and noncoding RNA. It is of great significance to clarify its role in regulating noncoding RNA, especially lncRNA. CLIP-seq sequencing screening results in this study showed that RBM10 can bind up to 357 lncRNAs at the genome-wide level, of which MALAT1 is the lncRNA that binds to RBM10 at the peak. Previous studies have shown that lncRNA can affect the phosphorylation of PI3K/AKT/mTOR pathway and thus affect tumor invasion and migration. Therefore, it is hypothesized that RBM10 may affect the phosphorylation of PI3K/AKT/mTOR pathway and the invasion and migration of NSCLC by binding and regulating MALAT1. As a cancer promoting factor, a large number of studies have shown that MALAT1 plays an important role in promoting the occurrence, development, invasion and migration of tumors. [21, 22] RBM10 is a nuclear protein existing in nucleosomes, and MALAT1 is also an intranuclear RNA, which means that they have the possibility of co-localization. We confirmed this hypothesis by RNA

immunocoprecipitation experiment. The results showed that RBM10 could directly bind MALAT1. QRT-PCR showed that overexpression of RBM10 could inhibit the level of MALAT1, and it was the opposite when RBM10 silenced.

MALAT1 can activate PI3K/AKT/mTOR pathway through ce-RNA mechanism, so as to promote the invasion and migration of tumor cells.[23, 24] Studies have shown that silencing high expression MALAT1 in oral cancer cells can reduce the level of p-AKT and the expression level of MMP-9; Using PI3K activator can eliminate the reduction of proliferation, migration and invasion of tongue cancer cells mediated by MALAT1 knockdown;[25] In osteosarcoma, MALAT1/miRNA-129-5p/Ret pathway can increase the expression of proteins downstream of Ret-AKT pathway and promote the proliferation, migration and invasion of osteosarcoma.[26] In order to determine whether RBM10 regulates PI3K/AKT/mTOR pathway through MALAT1, and then affects the invasion and migration of NSCLC, we transfected siRNA interference fragment to knock down MALAT1. When RBM10 was silenced, the phosphorylation levels of PI3K, AKT and mTOR proteins increased, and the ability of invasion and migration was also enhanced. After restoring MALAT1 in this cell line, the phosphorylation level of these proteins decreased, the total protein was not affected, and the invasion and migration ability of cells decreased. Therefore, it is concluded that RBM10 affects the phosphorylation of downstream PI3K/AKT/mTOR protein by regulating MALAT1, and finally affects the invasion and migration of NSCLC.

In recent years, more and more studies have proved that RNA binding proteins can participate in epigenetic regulation. M6A methylation is the most common and abundant post transcriptional epigenetic modification in eukaryotes, which is regulated by methyltransferases (writers), demethylases (erasers) and m6A binding protein (readers). Once these enzymes are abnormal, it will cause a series of diseases. MALAT1 is an lncRNA with multiple highly methylated sites. Recent studies have shown that the m6A methylation level of MALAT1 in lung cancer tissues is significantly higher than that in adjacent tissues. Mutant MALAT1 without m6A motif can significantly inhibit the metastatic potential of tumor cells in vivo and in vitro,[27] suggesting that inhibiting m6A methylation of MALAT1 can effectively inhibit the invasion and metastasis of lung cancer. In this study, SRAMP was used to predict and analyze the m6A methylation sites of MALAT1. Primers were designed for these sites. These sites were divided into three groups on the principle of full coverage. MeRIP-qPCR experiment was conducted to detect the m6A methylation level of each site respectively. The results of the methylation sites of the three groups were consistent. RBM10 overexpression can inhibit the m6A methylation level of three group sites, while RBM10 silencing can enhance the m6A methylation level, indicating that RBM10 can affect the m6A methylation of MALAT1, so as to realize the regulation of its epigenetics.

Studies have shown that there is an m6A "switch" in the structure of MALAT1. Under the catalysis of m6A methyltransferase METTL3, this "switch" is turned on. MALAT1 undergoes m6A methylation, resulting in its conformational change, from unstable RNA structure to stable hairpin structure, and controls the mature translation of other downstream mRNAs. Combined with our experimental results, it can be inferred that RBM10 reduces its structural stability by inhibiting m6A methylation of MALAT1, increases the proportion of unstable structures, and is prone to degradation, resulting in the reduction of measured

RNA level. As an RNA binding protein, RBM10 is not the key enzyme that regulates m6A process in the traditional sense. Some studies have pointed out that RBM15 can interact with m6A methyltransferase complex METTL3/METTL14/WTAP to regulate the dynamic level of m6A. [28] It is known that the gene sequence of RNA binding motif protein (RBM) family has high homology and is relatively conservative. Similarly, it is speculated that RBM10, which belongs to RNA binding motif family with RBM15, also has similar functions. In this study, the STRING database was also used to verify that there is an interaction between RBM10 and m6A methyltransferase and m6A reading protein, and RBM10 is closely related to the methylation process of m6A. Studies have shown that methyltransferase METTL3 is the key enzyme for MALAT1 m6A methylation in lung cancer.[29] Overexpression of METTL3 in lung cancer cells can increase the RNA expression level of MALAT1 and promote the invasion and metastasis of non-small cell lung cancer;[30] METTL3 can also enhance the stability of MALAT1 through m6A modification, so as to up regulate the expression of MALAT1. It can be seen that if the inhibition of METTL3 can be effectively regulated, m6A methylation of MALAT1 can be inhibited. Therefore, we first analyzed the protein interaction between METTL3 and RBM10 by STRING, and verified it by immunocoprecipitation at the molecular level. The results showed that there was an interactive binding relationship between METTL3 and RBM10, suggesting that RBM10 may regulate its m6A methylation process by recruiting METTL3 and combining MALAT1. (Fig. 7)

5. Conclusion

These results suggest that RBM10, as an RNA-binding protein, may inhibit m6A methylation of MALAT1 by recruiting METTL3, and affect phosphorylation of the downstream PI3K/AKT/mTOR pathway by binding and regulating MALAT1, ultimately affecting the invasion and migration of NSCLC.

Abbreviations

RBP: RNA Binding protein

RBM10: RNA Binding motif protein 10

MALAT1: Metastasis associated lung cancer transcript 1

LncRNA: long chain non-coding RNA

ceRNA: endogenous competitive RNA

NSCLC: non-small cell lung cancer

m6A: N6-methyladenosine methylation

RBM15: RNA Binding motif protein 15

IHC: immunohistochemistry

IP: Immunoprecipitation

RIP: RNA Binding Protein Immunoprecipitation

CLIP-seq: Crosslinking-immunprecipitation and high-throughput sequencing

LUAD: lung adenocarcinoma

LUSC: lung squamous cell carcinoma

METTL3: methyltransferase like 3

METTL14: methyltransferase like 14

PI3K: Phosphatidylinositol 3 Kinase

AKT: AKT Serine/Threonine Kinase

mTOR: Mammalian Target of Rapamycin

Declarations

Ethics approval and consent to participate

Human tissue arrays were provided from the second hospital of jilin university, which provided a certifcate statement to confrm the legitimacy of tissue resources.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and materials

The datasets generated/analyzed for this study can be found in the Gene Expression Omnibus (GSE48066) Majority of data generated or analyzed during this study are included in this published article and its supplementary information files. Non-included data may be obtained from the corresponding author upon reasonable request.

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KW contributed to the conception and design of the study. YC, SC, CT, XZ and MZ reviewed and analyzed the literature. YC produced the main draft of the manuscript. YC, XJ, YW and XD made figures. WR revised the manuscript. KW, WR and XD obtained funding for the study. All authors contributed to the article and approved the submitted version.

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Figures

Figure 1

Expression of RBM10 and MALAT1 in NSCLC tissues. (A) Immunohistochemistry results of RBM10 expression in normal and NSCLC tissues. Scale = 200μm. (B) Expression of RBM10 protein in NSCLC and adjacent tissues was detected by western blotting, quantified by Image J software. (C) QRT-PCR detection of MALAT1 expression in NSCLC and adjacent tissues (D) Overall survival and disease-free survival of cancer patients in GEPIA database. The differences between RBM10 and MALAT1 expression groups were compared. LUAD: lung adenocarcinoma; LUSC: lung squamous carcinoma (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001). P: para-cancer, C: cancer

Figure 2

RBM10 inhibits the invasion and migaration of NSCLC cells. (A) A549 and H460 cells were transfected with lentiviruses expressing RBM10 overexpression (oe-RBM10) and RBM10 RNA interference (sh-RBM10), compared with negtive control groups (NC-oe and NC-sh) respectively. Total protein extracts from the cells were analyzed by western blotting for RBM10 expression. (B) Transwell assays show that RBM10 inhibits the invasion of A549 and H460 cells. (C) Wound healing assays show that RBM10 inhibits the migration of A549 and H460 cells. Bar=200μm. (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001)

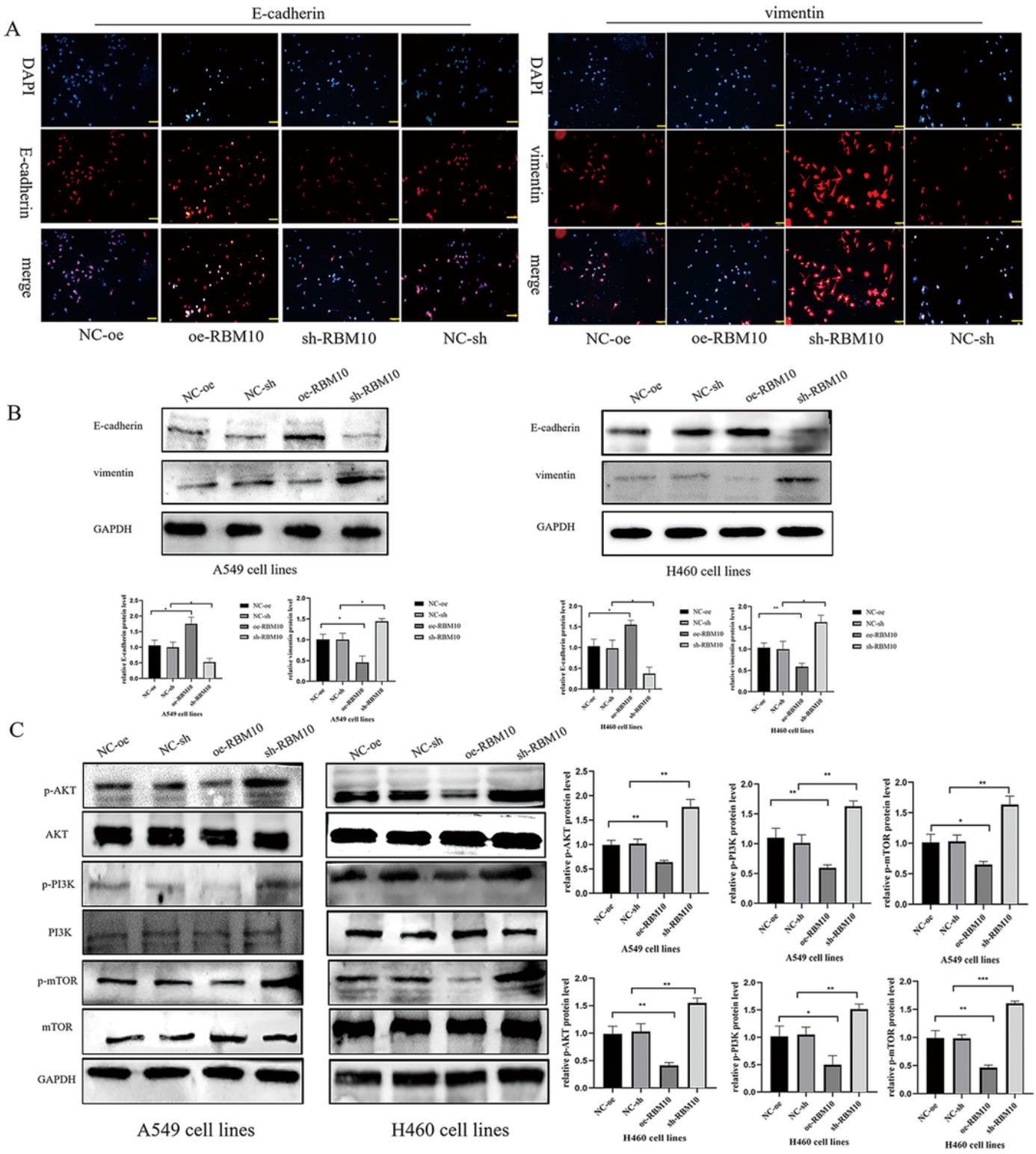

Figure 3

RBM10 affects the expression of EMT related proteins and the phosphorylation of PI3K / AKT / mTOR pathway.

(A) Immunofluorescence qualitative analysis of E-cadherin and vimentin protein. Scale bar = 50μm. (B) Western blotting qualitative analysis of E-cadherin and vimentin protein. (C) Western blotting analysis of

phosphorylation and total PI3K/AKT/mTOR pathway. Negtive control groups (NC-oe and NC-sh), RBM10 overexpression (oe-RBM10) and RBM10 RNA interference (sh-RBM10). (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001)

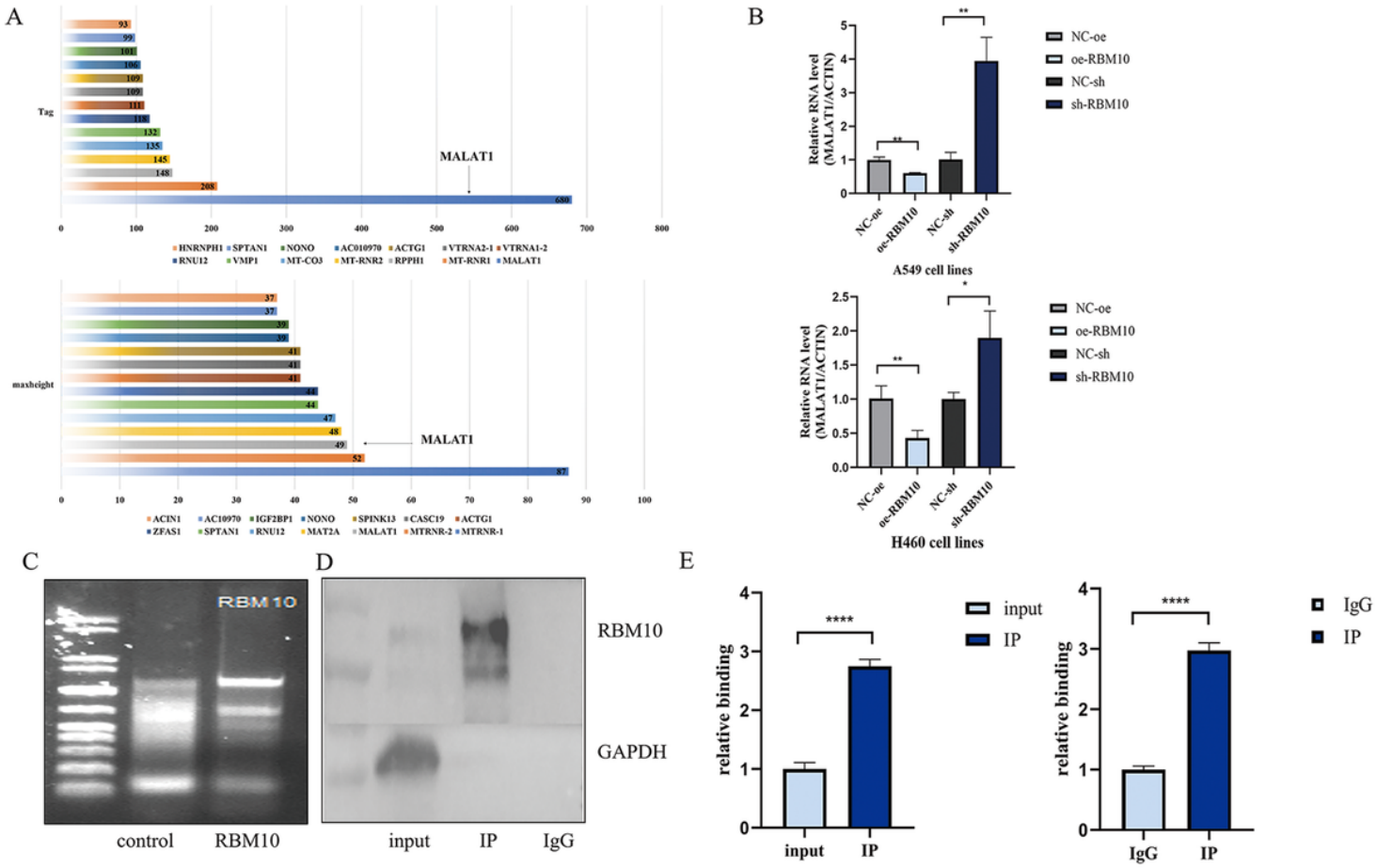

Figure 4

RBM10 directly binds and regulates the expression level of MALAT1. (A) CLIP-seq results of RBM10 genome-wide binding RNA. MALAT1 combined with tags is the first in 680 rows. The max-height of MALAT1 binding was 49, ranking third. (B) The level of MALAT1 was detected by qPCR in RBM10 transfected A549 and H460 cell lines. Negtive control groups (NC-oe and NC-sh), RBM10 overexpression (oe-RBM10) and RBM10 RNA interference (sh-RBM10). (C) RNA electrophoresis results after genome digestion in RIP experiment. Compared with the control group (undigested genome), the non-specific bands of RBM10 IP group (digested genome) were significantly reduced, and subsequent RIP experiments can be carried out. (D) Western blotting result of RIP. Input: After RNA fragmentation, before immunoprecipitation, take part of the broken chromatin as input control (no immunoprecipitation process) to eliminate background noise (excluding false positive peaks caused by high background expression level or some nonspecific binding); IP: RBM10 IP antibody precipitation was used; IgG: Homologous Rabbit anti IgG precipitation was used. (D) RIP-PCR was used to detect the relative binding

level of MALAT1 bound to RBM10 after RIP precipitation. Input group and IgG group were used as control. (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001)

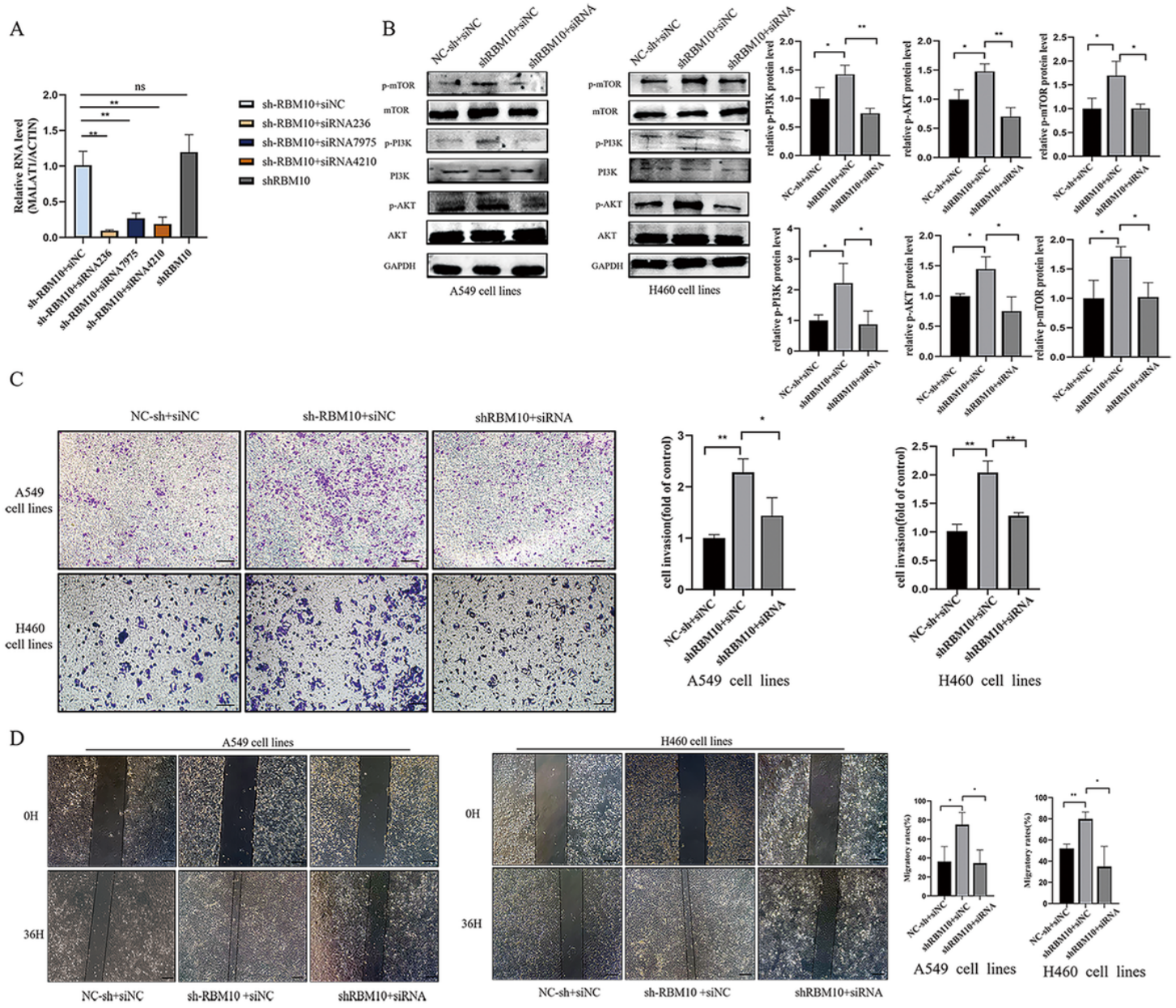

Figure 5

RBM10 regulates NSCLC invasion and migration and phosphorylation of PI3K/AKT/mTOR pathway through MALAT1. (A) QRT-PCR result of si-MALAT1 RNA in sh-RBM10 group. (B) Western blotting results of phosphorylation level of PI3K/AKT/mTOR pathway. Negtive control group (NC-sh+siNC), RBM10 RNA interference (sh-RBM10) and si-MALAT1-RNA in sh-RBM10 cell (sh-RBM10+siRNA). (C) Transwell assays show that RBM10 inhibits the invasion of A549 and H460 cells through MALAT1. (D) Wound healing assays show that RBM10 inhibits the migration of A549 and H460 cells through MALAT1. Bar=200μm. (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001)

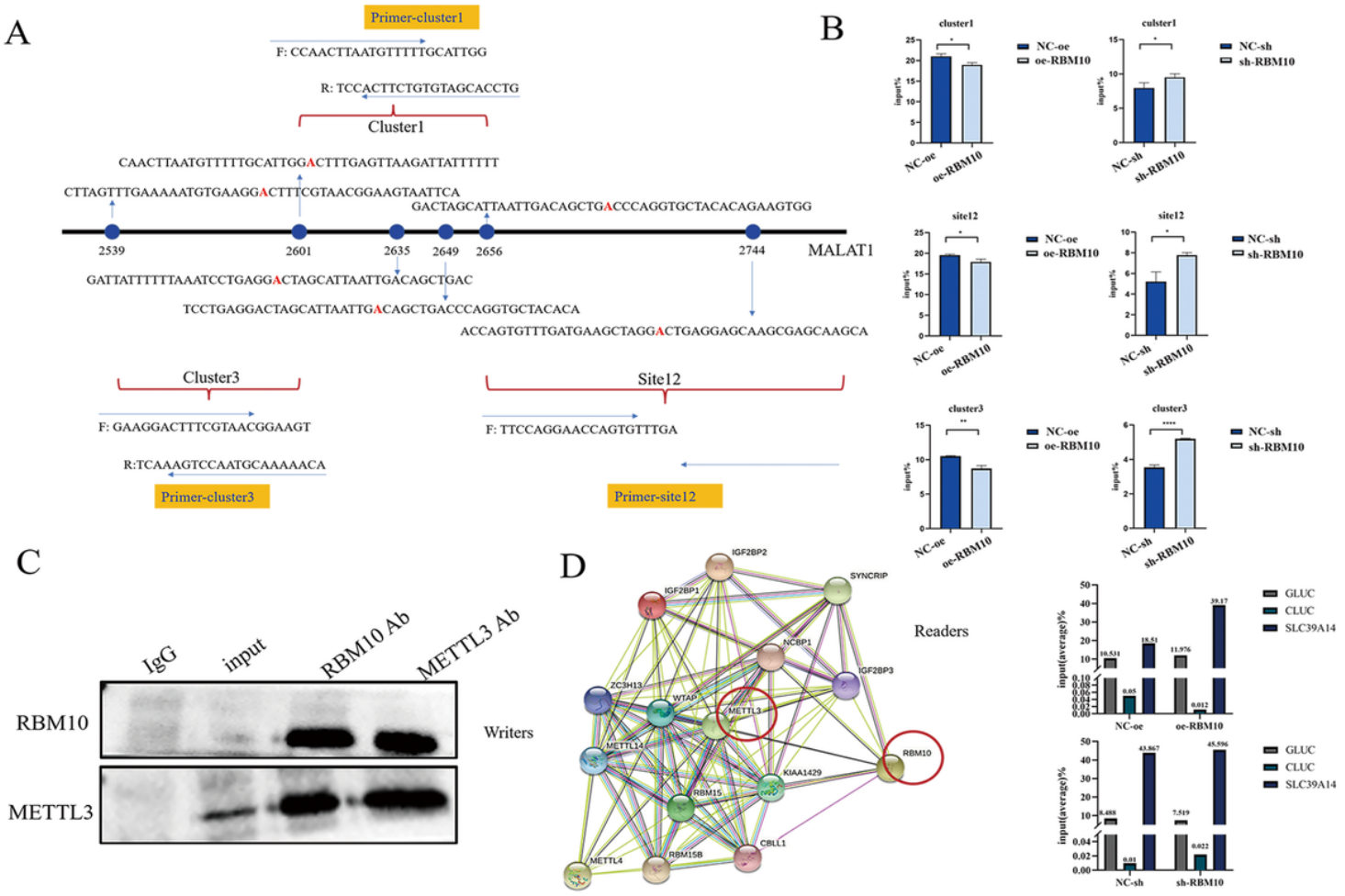

Figure 6

RBM10 inhibits m6A methylation of MALAT1. (A) M6A methylation site and primer design of MALAT1. There are six m6A methylation sites (2539, 2601, 2635,2649, 2656,2744) according to our testing. The six methylation sites were divided into three groups, (cluster1, site12 and cluster3) and three primers were designed to cover them. The red A means very high confidence m6A site. (B) The methylation level of MALAT1 (input%) at three methylation sites was compared with input. The component difference of MALAT1 input% between NC-oe and oe-RBM10 groups, NC-sh and sh-RBM10 groups was analyzed respectively. (C) Immunocoprecipitation result of RBM10 and METTL3. IgG: Precipitation with IgG antibody; RBM10 Ab: Precipitation with RBM10 antibody; METTL3 Ab: Precipitation with METTL3 antibody. (D) The interaction between METTL3 and RBM10 was analyzed by STRING protein interaction (PPI) database (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001)

Figure 7

RBM10 affects PI3K / AKT / mTOR pathway to inhibit the invasion and migration of NSCLC by regulating MALAT1 m6A methylation. In normal cell, the high content of RBM10 can recruiting METTL3 and then

directly bind with MALAT1 to form a complex, inhibit m6A methylation of MALAT1, reduce the RNA stability of MALTA1, then reduce the expression level of MALAT1, stimulate the phosphorylation of downstream PI3K / AKT / mTOR pathway and the expression of EMT related proteins, finally inhibit the invasion and migration process of NSCLC. In NSCLC cell, low content of RBM10 had the opposite effect of the above.

Supplementary Files

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