RBM24 Mediates Lymph Node Metastasis and Epithelial-Mesenchymal-Transition in Human Hypopharyngeal Squamous Cell Carcinoma Via Regulating Twist1

Yuhong Liu

First Affiliated Hospital of Chongqing Medical University

Tao Lu

First Affiliated Hospital of Chongqing Medical University

Min Pan

First Affiliated Hospital of Chongqing Medical University

Dan Yu

First Affiliated Hospital of Chongqing Medical University

Yanshi Li

First Affiliated Hospital of Chongqing Medical University

Guohua Hu (✉ hghcq@sina.com)

First Affiliated Hospital of Chongqing Medical University

Research Article

Keywords: HSCC, RBM24, Twist1, LN metastasis, EMT

Posted Date: December 16th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1167348/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Hypopharyngeal squamous cell carcinoma (HSCC) has the worst prognosis among head and neck tumours, and Lymph node (LN) metastasis mainly accounts for the poor prognosis. RBM24 (RNA Binding Motif Protein 24) regulates target RNA as an RNA binding protein involved in several cancers. However, its role in HSCC remains completely unknown. Here we attempt to explore the effects of RBM24 on HSCC.

Methods: RNA sequencing was conducted to find the differentially expressed genes in tumour tissues from HSCC patients with LN metastasis and without LN metastasis in our previous study. Expression of RBM24 in HSCC tissues was analyzed by qRT-PCR, western blot and immunohistochemistry. Cell proliferation was tested by CCK8 assay as well as Colony formation analysis. Cell migration and invasion capacity were estimated by transwell assay. The wound healing assay was also carried out to evaluate the motility of FaDu cells. QRT-PCR, western blot and immunofluorescence assays were conducted to detect the process of EMT. A popliteal lymph node metastasis model was constructed to explore the effect of RBM24 on HSCC in vivo.

Results: RBM24 was remarkably down-regulated in HSCC patients with LN metastasis, and low expression of RBM24 was inextricably linked to the poor prognosis. Knockdown of RBM24 facilitated the proliferation, migration and invasion of RBM24, whereas overexpression of RBM24 showed the opposite effects and suppressed the epithelial-mesenchymal-transition (EMT) process. Overexpression of Twist1 could reverse the inhibitory effects of RBM24 on motility and invasion of FaDu cells. The inhibitory effects of RBM24 on tumour growth and lymphatic metastasis in HSCC were demonstrated by the in vivo experiment as well.

Conclusions: These results indicated RBM24 was a suppressor gene and might inhibit EMT and LN metastasis in HSCC via regulating Twist1.

Background

Hypopharyngeal squamous cell carcinoma (HSCC) is a highly aggressive malignant tumour that accounts for nearly 0.4% of all cancers¹. Although rare, it has the worst prognosis compared with other kinds of head and neck tumours, and 5-year relative survival rate is 25%-45%²⁻⁵. Ho, A. S. et al. expound the number of metastatic lymph nodes is a principal independent factor closely related to mortality risk in hypopharyngeal cancer⁶. Despite the great improvements of diagnostic methods and therapeutic strategies, HSCC patients have developed advanced-stage disease and distant metastasis or cervical lymph node metastasis at the initial diagnosis, and LN metastasis occurs in 65%-80% of patients^{7, 8}. Hence, it is vital to elucidate the molecular mechanisms of cancer malignant progression in HSCC with LN metastasis to search for biomarkers for early screening and improve the accuracy of diagnosis. RNA binding proteins (RBPs) are almost involved in regulating the entire life of target RNA substrates, and they can be the core of gene expression network⁹. A census of human RBDs has uncovered 7.5% of the

~20,500 protein-coding genes in humans participate in gene expression regulation¹⁰. RBPs have been regarded as participants in tumorigenesis already¹¹. Conventional RBPs regulate transcripts by forming ribonucleoprotein (RNP) complexes with their RNA targets via RNA-binding domains (RBDs) such as the RNA recognition motif (RRM), the hnRNP K homology domain (KH domain), the double-stranded RNA-binding domain (dsRBD), S1 domains and others¹². RBM24 (RNA Binding Motif Protein 24), an RNA-binding protein containing a highly conserved RRM domain, comprises two submotifs, RNP1 and RNP2¹³. Previous researches have verified RBM24 is able to bind to G(A/U)GUG motif of target RNA^{14, 15}. Researches have shown RBM24 can promote differentiation of embryonic stem cells to cardiomyocytes by regulating alternative splicing¹⁶ and enhance myogenic differentiation by regulating the stability of myogenin mRNA transcripts via binding to its 3'-untranslated region (3'-UTR) mRNA¹⁷ in mice. RBM24 has also been found to play a tumour suppressor role in nasopharyngeal carcinoma(NPC)¹⁸, colorectal cancer (CRC)¹⁹ and liver cancer²⁰, meanwhile the exact function of RBM24 in HSCC tumorigenesis remains unclear.

Epithelial-mesenchymal transition (EMT) is known as a vital process that participates in tumour metastasis and is closely associated with tumour progression. It endows tumour cells with the ability to invade adjacent tissues and metastasize²¹. Twist1, a main regulator of EMT, is a member of the basic helix-loop-helix proteins family. Up-regulation of Twist1 is reported to participate in the malignant progression of HSCC and associated with poor prognosis^{22, 23}. It has been indicated Twist1 overexpression can decrease the chemosensitivity of FaDu cells to taxol²⁴ while its effect on LN metastasis of HSCC is uncertain.

In the present study, we identified RBM24 was down-regulated in HSCC patients with LN metastasis for the first time. We proved RBM24 could inhibit HSCC tumour proliferation and metastasis both in vitro and in vivo. Furthermore, we demonstrated Twist1 was a downstream target of RBM24 and Twist1 overexpression could rescue the inhibiting effects of RBM24 on migration, invasion and EMT of FaDu cells. It indicated RBM24 might restrain LN metastasis by targeting Twist1.

Materials And Methods

Patients and Tissue Samples

We obtained surgical specimens from patients diagnosed with HSCC in the First Affiliated Hospital of Chongqing Medical University between 2015 and 2019. The inclusion criteria were as follows: (1) The pathological diagnosis was hypopharyngeal squamous cell carcinoma. (2) Laryngectomy was performed for the first time. (3) All patients had complete follow-up data and underwent electronic nasopharyngoscope, CT or MRI examination every six months after the initial operation. The exclusion criteria were as follows: (1) Patients underwent preoperative radiotherapy, chemotherapy or targeted therapy. (2) Patients diagnosed with other types of tumours. The pathological diagnosis and tumour grading of these cases were identified by two experienced pathologists. Finally, 57 patients diagnosed

with HSCC who met the criteria were involved in this study. The study was performed in accordance with the Declaration of Helsinki, and the ethics committee of the first affiliated hospital of Chongqing Medical University approved this study. Written informed consent forms were obtained from the patients before they underwent surgery.

Immunohistochemistry and scoring

The paraffin-embedded tissue sections (4µm) were deparaffinized in xylene and rehydrated in graded ethanol. Sections were heated in citrate buffer for 15 minutes at 100°C for antigen retrieval. Next, the slides were rinsed in phosphate buffer saline (PBS) and were treated with Peroxidase Block at 37°C for 10 minutes. After being blocked with 10% normal goat serum at 37°C for 15 minutes to minimize nonspecific staining, slides were incubated with the primary RBM24 antibody (1:200, ab94567, Abcam, UK) overnight at 4°C. The next day, slides were treated with HRP-conjugated polyclonal goat secondary antibody for 15 minutes, and antigen-antibody reaction signals were detected with Diaminobenzidine (DAB) reagents (ZSGB-BIO, China) at room temperature for 1 minute. Finally, slides were counterstained with hematoxylin for 20 seconds. The slides using PBS instead of primary antibody were negative control. And all of the slides were estimated by two experienced pathologists. IHC staining scores depended on both the proportion of RBM24-positive tumour cells and the staining intensity. The proportion of RBM24-positive tumor cells were scored as 0 (0–5%), 1 (5–25%), 2 (25–50%), 3 (50–75%), and 4 (75–100%). The staining intensity was scored as 0 (negative staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). The final IHC scores were the multiplication of the two scores. ROC curve was used to analyze IHC scores to distinguish the protein level of RBM24 and the cutoff value was 4. Scores < 4 were defined as low expression while scores $\geq$ 4 were considered as high expression.

Cell line and culture

The human hypopharyngeal carcinoma cell line FaDu was purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China) and cultured in minimal essential medium (MEM) (Boster, USA) supplemented with 10% fetal bovine serum (PAN, Germany) and antibiotics (100 U/ml of penicillin and streptomycin) in an incubator containing 5% CO₂ at 37°C.

Lentivirus transductions

To overexpress RBM24, lentiviral particles (OE-RBM24 and Control) containing green fluorescent protein (GFP) were obtained from GeneChem Corporation (Shanghai, China). Fadu cells were seeded into a six-well plate then infected by the lentiviral particles with a multiplicity of infection (MOI) of 10 when the cells were at 20–40% confluency. After 48–72h of lentivirus infection, infected cells were selected with 2 µg/ml puromycin (Beyotime, China). Then the cells were collected and examined by qRT-PCR and Western blot.

SiRNA transfection

To knockdown RBM24, siRNA oligonucleotide (Si-RBM24, 5'- GAG CUG CAU ACG CAC AAU ATT- 3', 5'-UAU UGU GCG UAU GCA GCU CTT'-3') against human RBM24 and scrambled siRNA (si-NC, sense, 5'-UUC UCC GAA CGU GUC ACG UTT-3', antisense, 5'-ACG UGA CAC GUU CGG AGA ATT-3') were acquired from GenePharma (Shanghai, China). FaDu cells inoculated onto a six-well plate were transfected with these siRNAs by Lipofectamine RNAi MAX (Invitrogen, USA) following the manufacturer's instructions. QRT-PCR and western blot were used to test the knockdown efficiency.

Plasmids transfection

The control plasmids (PCMV) and Twist1 plasmids (OE-Twist1) were obtained from Tsingke Biotechnology Co., Ltd. (Nanjing, China). The plasmids were transfected into FaDu cells with Lipofectamine 3000 (Invitrogen, USA).

Western blot (WB) and antibodies

The total protein extracts of tissues and FaDu cells were obtained using the total protein extraction kit (China KeyGen biotechnology company). The Protein BCA Assay Kit (Beyotime, China) was used to evaluate the protein concentrations. An equal amount of protein mix was separated through 10% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Biosharp, China). The membranes were blocked with blocking buffer (Beyotime, China) for 15 minutes at room temperature and incubated with anti-RBM24(1:1000, ab94567, Abcam, UK), anti-Twist1 (1:1000, AF4009, Affinity, China), anti-E-cadherin (1:1000, #3195, Cell Signaling Technology), anti-N-cadherin (1:1000, #13116, Cell Signaling Technology), anti-Vimentin (1:5000, ab92547, Abcam UK), anti-GAPDH (1:3000, AF1186, Beyotime, China) overnight at 4°C. The secondary goat anti-rabbit IgG (1:5000, A0208, Beyotime, China) was used to incubate the membranes for 1 hour at room temperature. Then the ChemiDOCTMXRS+ (Bio-Rad) was used to detect specific proteins.

Quantitative Real-Time PCR Analysis (qRT-PCR)

The Total RNA Kit I (Omega, USA) was applied to extract total RNA. The PrimeScript RT Kit (Takara, China) was utilized to obtain complementary DNA and qRT-PCR was conducted with SYBR PrimeScript RT-PCR Kit (Takara, China). The primer sequences were: RBM24 forward, 5'-GAA CCT GGC ATA CTT AGG AGC A-3', reverse, 5'-AGG TCT TTG TAT AAG GGC TGG A-3'; E-cadherin forward, 5'-TGC CCA GAA AAT GAA AAA GG-3'; reverse, 5'-GTG TAT GTG GCA ATG CGT TC-3'. N-cadherin forward, 5'-GAC AAT GCC CCT CAA GTG TT-3'; reverse, 5'-CCA TTA AGC CGA GTG ATG GT-3'; Vimentin forward, 5'-GAG AAC TTT GCC GTT GAA GC-3'; reverse, 5'-GCT TCC TGT AGG TGG CAA TC-3'. Twist1 forward, 5'-ACA AGC TGA GCA AGA TTC AGA CC-3'; reverse, 5'-TCC AGA CCG AGA AGG CGT AG-3'; Snail, forward, 5'-GCG AGC TGC AGG ACT CTA AT-3'; reverse, 5'- CCT CAT CTG ACA GGG AGG TC-3'; Slug, forward, 5'- TGA TGA AGA GGA AAG ACT ACAG-3'; reverse, 5'-GCTCACATATTCCTTGTCACAG-3'; ZEB1, forward, 5'- TGC ACT GAG TGT GGA AAA GC-3'; reverse, 5'- TGG TGA TGC TGA AAG AGA CG-3'; GAPDH, forward, 5'-GGA GTC CAC TGG CGT CTT CA-3'; reverse, 5'-GTC ATG AGT CCT TCC ACG ATA CC-3'.

Cell Proliferation Assay

The cell growth ability was measured using Cell Counting Kit-8 (CCK-8, GlpBio, USA), a cell proliferation reagent. After adding 10 microliters (10 μ l) of CCK-8 to each well, then the 96-well plate was incubated at 37°C for 1 hour. Finally, the absorbance was evaluated at 450 nm to collect uninterrupted data for 96 hours.

Colony Formation Analysis

1,000 FaDu cells were inoculated onto each well of a six-well plate and incubated in a medium containing 10% FBS at 37°C and 5% CO₂ for 2 weeks. After fixing the cells with methanol and staining the cells with 0.5% crystal, colonies composed of at least 50 cells were counted.

Transwell Migration and Invasion Assay

To estimate the cell migration and invasion assays of FaDu cells, transwell assays were performed with 8 μ m pore-chambers (Corning, USA), with or without Matrigel (BD Biosciences, Bedford, MA, USA) following the manufacture's instruction. 200 μ l serum-free MEM containing 5×10^4 were seeded into the upper chamber. 600 μ L MEM medium supplemented with 20% FBS were added into the lower chamber. After 48 hours, the cells were fixed with methanol and stained with 0.5% crystal violet. Finally, the number of stained cells were counted with a microscope.

Wound healing scratch assay

FaDu cells were cultured in six-well plates and 200 μ l sterile pipette tips were used to create linear scratches in the confluent monolayers of cells. Cellular debris was washed away using PBS. Then the wound closure was observed with a microscope at 0 hour and 24 hours after injury.

Immunofluorescence (IF) Assay

FaDu cells were inoculated onto glass coverslips in a 24-well plate, fixed with 4% paraformaldehyde for 15 minutes, and permeabilized with 0.5% Triton X-100 for 20 minutes. Then the coverslips were incubated with anti-E-cadherin (1:100, #3195, Cell Signaling Technology), anti-N-cadherin(1:100, #13116, Cell Signaling Technology), anti-Vimentin (1:500, ab92547, Abcam,) overnight at 4°C after having been blocked with goat serum (Beyotime, China) for 1 hour. The next day, coverslips were incubated using the fluorescent secondary antibody (1:250, ab150078, Abcam, UK) for 1 hour and DAPI (Beyotime, Shanghai, China) for 5 minutes at room temperature. To retard the fluorescence quenching, antifade mounting medium was added to the coverslips. Finally, the coverslips were observed and captured with a laser scanning confocal microscope.

Animal Experiments

BALB/c male nude mice (5 weeks old; Tengxin, China) were chosen to establish popliteal lymph node metastasis models and fed under specific pathogen-free conditions in the Animal Laboratory of Chongqing Medical University. The mice were randomly divided into 2 groups (5 mice per group) and injected with 2×10^6 FaDu-Control or FaDu-OE-RBM24 cells suspended in 30 μ l PBS in their footpads. Tumour size and weight of the mice were measured every 5 days using a caliper. Tumour growth and LN metastasis were observed by the small animal in vivo imaging (IndiGo 2.0.5.0 system, Berthold Technologies, Germany) 15 days after cell implantation. All the mice were sacrificed 25 days later, the primary tumour tissues and lymph nodes were removed and paraffin-embedded. Tumour volumes were calculated in accordance with the formula $0.5 \times \text{length} \times \text{width}^2$ and the tissues were analyzed by immunohistochemistry (IHC) of anti-RBM24(1:200, ab94567, Abcam, UK), anti-Twist1(1:100, AF4009, Affinity), anti-E-cadherin(1:400, #3195, Cell Signaling Technology), anti-Vimentin(1:250, ab92547, Abcam, UK). The lymph nodes were stained with hematoxylin and eosin (H&E). All of the animal experiments received approval from the ethics committee of the first affiliated hospital of Chongqing Medical University.

Statistical Analysis

SPSS 22.0 and GraphPad Prism 8.0 were used for statistical analyses. A Student's t-test or the Mann-Whitney U-test was selected to assess significant differences between two groups while one-way ANOVA was applied to three or more groups. The correlation between expression of RBM24 in immunohistochemistry and clinicopathological features was evaluated by Pearson's chi-square test or Fisher's exact test. Kaplan-Meier & log-rank analysis was performed to evaluate overall survival (OS). All results are shown as mean $\pm$ standard error of mean (SEM). P-value < 0.05 was considered statistically significant.

Results

RBM24 was down-regulated in HSCC patients with lymph node metastasis.

We conducted RNA sequencing to find the differentially expressed genes (DEmRNA) in HSCC patients with LN metastasis and without LN metastasis in our previous study^{25, 26}. And RBM24 was one of the down-regulated genes in the LN metastasis group. Then we expanded the sample size to detect expression of RBM24 at the mRNA level as well as the protein level. Using 8 pairs of tumour tissues (including 8 tissues from patients with LN metastasis and 8 tissues from patients without LN metastasis) and corresponding adjacent normal tissues, we identified the lower mRNA level in the LN metastasis group than in the normal group ($p < 0.01$) as well as than in the HSCC patient group without LN metastasis ($p < 0.05$) (Fig. 1 a). Correspondingly, we identified the protein levels of RBM24 were lower in the LN metastasis group than in the other two groups through WB analysis in 5 pairs of tumour tissues (including 5 tissues from patients with LN metastasis and 5 tissues from patients without LN metastasis) and corresponding adjacent normal tissues ($p < 0.05$) (Fig. 1b). We also conducted IHC to determine the expression of RBM24 in tissues. The different staining of RBM24 in HSCC tissues was exhibited in Fig.

1c. The frequency of low expression of RBM24 in tissues from patients with LN metastasis showed a greater proportion (25/41[61.0%]) than in those without LN metastasis (14/16[12.5%]) (**Table 1**). The results indicated RBM24 was down-regulated in HSCC patients with LN metastasis. Furthermore, we assessed the association between the expression of RBM24 and clinical features in 57 HSCC patients. Results showed the expression of RBM24 was connected with tumour stage ($p<0.01$) and lymph node stage ($p<0.001$) while was not connected with age, gender or pathological stage (**Table 2**). Kaplan-Meier survival analysis demonstrated patients with low expression of RBM24 had significantly poor overall survival (hazard ratio [HR]=3.114; 95% confidence interval [CI], 1.223–7.931, $p<0.05$) (Fig. 1d). These results indicated RBM24 was down-regulated in HSCC tissues with LN metastasis and was closely related to poor prognosis.

Table 1 The immunohistochemical expression of RBM24 in HSCC patients with lymphatic metastasis and without metastasis

Tissue	Number of patients	Expression of RBM24		P value [#]
		High	Low	
Lymphatic metastasis	41	16(39.0%)	25(61.0%)	<0.001
Non-lymphatic metastasis	16	14(87.5%)	2(12.5%)	
#P values are from χ2 test or Fisher's exact test and were statistically significant when< 0.05.				

Inhibition of RBM24 Promoted Proliferation, Migration, and Invasion of FaDu Cells

To explore the biological function of RBM24 in Fadu cells, qRT-PCR and western blot were performed to verify the RBM24 expression. FaDu cells were transfected with RBM24-siRNAs (siRNA#1, siRNA#2, and siRNA#3) as well as control siRNA(Si-NC). The qRT-PCR and western blot results showed that siRNA#3 (Si-RBM24) was the most efficient to knock down RBM24 (Supplementary Fig. 1a and b, Fig. 2a and b), thus it was selected for further experiments. We found inhibition of RBM24 promoted the proliferation of FaDu cells through CCK-8 staining and colony formation assays (Fig. 2c and d). Moreover, knockdown of RBM24 enhanced migration and invasion (Fig. 2e) and wound healing (Fig. 2f) of FaDu cells.

Table 2 Relationship between RBM24 expression and clinicopathological characteristics of 57 HSCC patients

Category	Number of patients	Expression of RBM24		P value [#]
		High	Low	
Age(years old)				0.63
<60	23	13	10	
≥60	34	17	17	
Gender				1
male	56	29	27	
female	1	1	0	
T stage				<0.01
T1+T2	11	10	1	
T3+T4	46	20	26	
N stage				<0.001
N0+N1	24	20	4	
N2+N3	33	10	23	
Pathological stage				0.82
high differentiation	14	7	7	
moderate or poor differentiation	43	23	20	
#P values are from χ^2 test or Fisher's exact test and were statistically significant when < 0.05.				
Abbreviations: T stage, tumour stage; N stage, lymph node stage				

Overexpression of RBM24 Inhibited Proliferation, Migration, and Invasion of FaDu Cells We established a stable RBM24-overexpression FaDu cell line infected with lentiviral particles, and the efficiency of overexpression was tested by qRT-PCR (Fig. 3a) and western blot (Fig. 3b) assays. Results showed overexpression of RBM24 reduced the proliferation (Fig. 3c and d) and inhibited the mobility (Fig. 3e and f) of FaDu cells. These results revealed RBM24 might negatively regulate proliferative and invasive behaviours of FaDu cells.

Overexpression of RBM24 Down-regulated Twist1 and Inhibited EMT of FaDu Cells EMT is generally considered as an important process in tumour metastasis, and TGF β has been widely reported as a potent inducer of EMT²⁷. Our results indicated that loss of RBM24 may induce the malignant progress of hypopharyngeal squamous cell carcinoma (HSCC), so we turned to explore the association between

RBM24 and TGF β -induced EMT. We induced EMT in FaDu cells with 20 ng/ml TGF β for 48 hours and then tested the mRNA levels of EMT-inducing transcription factors (EMT-TFs) to find which was in response to RBM24 overexpression in FaDu cells. Finally, Twist1 was identified to be the downstream effector of RBM24, while Snail, Slug or ZEB1 did not change significantly (Fig. 4a). We also confirmed RBM24 overexpression also down-regulated the protein level of Twist1 (Fig. 4c). Correspondingly, we also found the mRNA and protein levels of E-cadherin increased while the levels of N-cadherin and Vimentin decreased in the RBM24-overexpression FaDu cells (Fig. 4b and c), indicating RBM24 overexpression restrained TGF β -induced EMT. Furthermore, overexpression of RBM24 resulted in decreasing E-cadherin and increasing N-cadherin and Vimentin levels evaluated by confocal immunofluorescence assay (Fig. 4d). These results suggested RBM24 could inhibit the process of TGF β -induced EMT and down-regulate expression of Twist1.

Overexpression of Twist1 Reversed the Inhibiting Effects of RBM24 on Cell Invasion, Migration and EMT of FaDu cell.

Since Twist1 played an essential role in HSCC progression, we determined whether Twist1 was necessary for the functions of RBM24 in HSCC cells by transfecting FaDu-OE-RBM24 cells with Twist1 plasmids (Fig. 5a and b). The results indicated overexpression of RBM24 suppressed cell migration, invasion and EMT while the inhibition effects were reversed by overexpression of Twist1 (Fig. 5c-e). It demonstrated overexpression of RBM24 could suppress cell motility, invasion capacity and EMT via negatively regulating Twist1.

Overexpression of RBM24 Suppressed HSCC Tumor Growth and Lymph Node Metastasis In Vivo

To explore the effects of RBM24 expression on hypopharyngeal tumour growth and lymph node metastasis in vivo, we injected FaDu cells stably expressing RBM24 (FaDu-OE-RBM24) or an empty vector (FaDu-Control) into the footpads of BALB/c male nude mice (Fig. 6a). As Fig. 6b showed, clear fluorescent signals were detected at the footpads in both groups. However, the fluorescence signal only emerged in the popliteal fossa of the mouse injected with FaDu-Control cells, representing LN metastasis. As the results showed in Fig 6c and d, tumours in mice injected with FaDu-OE-RBM24 cells grew more slowly compared with those injected with FaDu-Control cells. And the volume of tumours was lower in the OE-RBM24 group comparatively. The H&E assay showed stable overexpression of RBM24 resulted in fewer metastatic tumour cells in the lymph node (Fig. 6e). Meanwhile, 20% (1/5) of the mice implanted with FaDu-OE-RBM24 cells developed lymph node metastasis, whereas 75% (3/4) developed lymph node metastasis in the FaDu-Control group (Fig. 6f). Moreover, IHC assay showed the expression level of E-cadherin and RBM24 was higher while the level of Vimentin and Twist1 was lower in tumours induced by FaDu-OE-RBM24 cells than that induced by FaDu-Control cells (Fig. 6g). Hence, it was reasonable to speculate that overexpression of RBM24 could down-regulate Twist1 in vivo, which was also reflected in vitro.

Discussion

RNA binding proteins (RBPs) play vital roles in regulating RNA metabolic pathways, including transcription, alternative splicing, modification, polyadenylation, stability, subcellular localization, translation and decay^{11, 28}. Due to their significant importance in gene regulation, it has been reported that aberrant expression of RBPs or mutations in RBPs as well as their binding sites with RNA targets are involved in diverse human diseases, including cancers²⁹. Recently, a review has revealed RBPs participate in tumour metastasis by regulating mechanotransduction³⁰.

RBM24, an RNA binding protein, belongs to the RRM family. RBM24 has been identified as a participant in the p53 pathway through the mutual interaction with p53 and regulating p21 mRNA stability in different kinds of human cancer cell lines^{13 31}. It has been reported RBM24 also regulates p63 expression via mRNA stability³². Notably, RBM24 is found to inhibit cancer progression in several tumors¹⁸⁻²⁰. However, there is still a lack of understanding of its role in tumor initiation and progression, especially in hypopharyngeal carcinoma. In the early work, our team has carried out RNA sequencing to explore genes with altered expression in HSCC patients with or without LN metastasis, and RBM24 was one of the down-regulation genes. In the present study, we first verified RBM24 was down-regulated in HSCC patients with LN metastasis. Moreover, the lower expression of RBM24 was associated with several clinicopathological features and worse overall survival. It indicated that RBM24 might be a potential target for both early diagnosis and therapy in HSCC. To determine the biological function of RBM24 in vitro, we utilized siRNAs and lentiviral particles to regulate the expression of RBM24 in FaDu cells. And the results showed RBM24 could inhibit cell proliferation, migration, invasion abilities as well as EMT process in vitro. Based on these results, we suspected RBM24 might be a tumour suppressor to inhibit HSCC progression.

HSCC patients often have developed cervical lymph node metastasis at their initial diagnosis, leading to treatment failure and relatively poor overall survival^{4, 33-35}. Thus, it is particularly urgent to study the mechanism of HSCC tumor progression as well as LN metastasis. EMT has been widely reported to play crucial roles in the cancer process and is characterized by the loss of polarity of the epithelial cells and the conversion of epithelial cells to a mesenchymal phenotype³⁶, which endows the cancer cells with the ability to escape from the primary environment and develop distant metastasis. And EMT is defined by the decreased expression of epithelial marker E-cadherin and increased expression of mesenchymal marker N-cadherin and Vimentin³⁷. Recent studies have demonstrated the close associations between EMT and lymph node metastasis^{38, 39}. As we know, EMT is mainly regulated by EMT-inducing transcription factors (EMT-TFs), and Twist1 is one of the core EMT-TFs²⁷. Over the past years, Twist1 has been uncovered to be an oncogene, and its abnormal overexpression in various tumours facilitates cancer initiation, progression and metastasis. Twist1 is able to promote tumour process by enhancing the proliferation and suppressing apoptosis of cancer cells, increasing cancer cell resistance to chemotherapy, up-regulating cancer stem cell (CSC) population, and driving cancer cell invasion and metastasis^{40, 41}. Considering the significant importance of EMT in tumor metastasis and a previous study discovered RBM24 overexpression upregulated E-cadherin expression while downregulated the expression of Vimentin in human colorectal cancer HCT116 cells¹⁹, we turned to investigate the

interactions between RBM24 and TGF β -induced EMT. Our study had confirmed that RBM24 could regulate the expression of several typical markers of EMT and Twist1 in HSCC FaDu cells. This suggested RBM24 might reduce TGF β -induced EMT and HSCC LN metastasis by targeting Twist1. To verify the hypothesis, we carried out rescue experiments by overexpressing Twist1 in FaDu cells. The results indicated cell motility, invasion ability as well as the EMT process could be reversed by Twist1 overexpression. In addition, we found RBM24 overexpression could suppress HSCC tumour growth and LN metastasis in animal experiments. Meanwhile, IHC staining showed Twist1 and several EMT markers changed correspondingly. Thus, it was feasible to speculate that overexpression of RBM24 might inhibit HSCC tumour growth and it might reduce LN metastasis by restraining Twist1 expression. However, it is still necessary to conduct further studies to verify whether RBM24 can combine with Twist1 and elucidate the specific mechanism. As well, the detailed mechanisms of how they regulate EMT and LN metastasis after RBM24 combining with Twist1 need further research.

In summary, RBM24 serves as a novel tumour suppressor gene and suppresses the LN metastasis and EMT process by downregulating the level of Twist1 in HSCC. Therefore, RBM24 and Twist1 may be potential biomarkers for diagnosis and therapeutic targets for HSCC patients with LN metastasis.

Declarations

Acknowledgement

We would like to thank our lab colleagues for their help in this study.

Funding

This study was financially supported by the National Natural Science Foundation of China (No. 81902776), the Natural Science Foundation of Chongqing (cstc2019jcyj-msxm0325) and the Natural Science Foundation of Chongqing (2020MSXM091).

Authors' contributions

YL, MP and GH designed the research. YL operated the experiments, analyzed the data and wrote the manuscript. TL, DY and YL revised this paper. All authors read and approved the final manuscript.

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Ethics approval was approved by the ethics committee of the first affiliated hospital of Chongqing Medical University. The animal study was approved by the ethics committee of the first affiliated hospital of Chongqing Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R. L.; Torre, L. A.; Jemal, A., Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* **2018**, *68* (6), 394-424.
2. Gatta, G.; Capocaccia, R.; Botta, L.; Mallone, S.; De Angelis, R.; Ardanaz, E., et al., Burden and centralised treatment in Europe of rare tumours: results of RARECAREnet—a population-based study. *The Lancet Oncology* **2017**, *18* (8), 1022-1039.
3. Tian, S.; Li, Q.; Li, R.; Chen, X.; Tao, Z.; Gong, H., et al., Development and Validation of a Prognostic Nomogram for Hypopharyngeal Carcinoma. *Front Oncol* **2021**, *11*, 696952.
4. Grasl, S.; Janik, S.; Parzefall, T.; Formanek, M.; Grasl, M. C.; Heiduschka, G., et al., Lymph node ratio as a prognostic marker in advanced laryngeal and hypopharyngeal carcinoma after primary total laryngopharyngectomy. *Clin Otolaryngol* **2020**, *45* (1), 73-82.
5. Garneau, J. C.; Bakst, R. L.; Miles, B. A., Hypopharyngeal cancer: A state of the art review. *Oral oncology* **2018**, *86*, 244-250.
6. Ho, A. S.; Kim, S.; Tighiouart, M.; Gudino, C.; Mita, A.; Scher, K. S., et al., Association of Quantitative Metastatic Lymph Node Burden With Survival in Hypopharyngeal and Laryngeal Cancer. *JAMA Oncol* **2018**, *4* (7), 985-989.
7. Eckel, H. E.; Bradley, P. J., Treatment Options for Hypopharyngeal Cancer. *Advances in oto-rhino-laryngology* **2019**, *83*, 47-53.
8. Wang, H.; Wu, R.; Huang, X.; Qu, Y.; Wang, K.; Liu, Q., et al., The Pattern of Cervical Lymph Node Metastasis and Risk Factors of Retropharyngeal Lymph Node Metastasis Based on Magnetic Resonance Imaging in Different Sites of Hypopharyngeal Carcinoma. *Cancer Manag Res* **2020**, *12*, 8581-8587.
9. Babitzke, P.; Lai, Y. J.; Renda, A. J.; Romeo, T., Posttranscription Initiation Control of Gene Expression Mediated by Bacterial RNA-Binding Proteins. *Annu Rev Microbiol* **2019**, *73*, 43-67.
10. Gerstberger, S.; Hafner, M.; Tuschl, T., A census of human RNA-binding proteins. *Nat Rev Genet* **2014**, *15* (12), 829-45.
11. Pereira, B.; Billaud, M.; Almeida, R., RNA-Binding Proteins in Cancer: Old Players and New Actors. *Trends in cancer* **2017**, *3* (7), 506-528.

12. Lunde, B. M.; Moore, C.; Varani, G., RNA-binding proteins: modular design for efficient function. *Nature reviews. Molecular cell biology* **2007**, *8* (6), 479-90.
13. Jiang, Y.; Zhang, M.; Qian, Y.; Xu, E.; Zhang, J.; Chen, X., Rbm24, an RNA-binding protein and a target of p53, regulates p21 expression via mRNA stability. *J Biol Chem* **2014**, *289* (6), 3164-75.
14. Ray, D.; Kazan, H.; Cook, K. B.; Weirauch, M. T.; Najafabadi, H. S.; Li, X., et al., A compendium of RNA-binding motifs for decoding gene regulation. *Nature* **2013**, *499* (7457), 172-7.
15. Liu, J.; Kong, X.; Zhang, M.; Yang, X.; Xu, X., RNA binding protein 24 deletion disrupts global alternative splicing and causes dilated cardiomyopathy. *Protein Cell* **2019**, *10* (6), 405-416.
16. Zhang, T.; Lin, Y.; Liu, J.; Zhang, Z. G.; Fu, W.; Guo, L. Y., et al., Rbm24 Regulates Alternative Splicing Switch in Embryonic Stem Cell Cardiac Lineage Differentiation. *Stem Cells* **2016**, *34* (7), 1776-1789.
17. Jin, D. H.; Hidaka, K.; Shirai, M.; Morisaki, T., RNA-binding motif protein 24 regulates myogenin expression and promotes myogenic differentiation. *Genes To Cells* **2010**, *15* (11), 1158-1167.
18. Hua, W. F.; Zhong, Q.; Xia, T. L.; Chen, Q.; Zhang, M. Y.; Zhou, A. J., et al., RBM24 suppresses cancer progression by upregulating miR-25 to target MALAT1 in nasopharyngeal carcinoma. *Cell Death Dis* **2016**, *7* (9), e2352.
19. Xia, R. M.; Liu, T.; Li, W. G.; Xu, X. Q., RNA-binding protein RBM24 represses colorectal tumorigenesis by stabilising PTEN mRNA. *Clin Transl Med* **2021**, *11* (10), e383.
20. Choi, J. H.; Kwon, S. M.; Moon, S. U.; Yoon, S.; Shah, M.; Lee, B. G., et al., TPRG1-AS1 induces RBM24 expression and inhibits liver cancer progression by sponging miR-4691-5p and miR-3659. *Liver Int* **2021**, *41* (11), 2788-2800.
21. Lee, J. M.; Dedhar, S.; Kalluri, R.; Thompson, E. W., The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *J Cell Biol* **2006**, *172* (7), 973-81.
22. Way, T. D.; Huang, J. T.; Chou, C. H.; Huang, C. H.; Yang, M. H.; Ho, C. T., Emodin represses TWIST1-induced epithelial-mesenchymal transitions in head and neck squamous cell carcinoma cells by inhibiting the beta-catenin and Akt pathways. *Eur J Cancer* **2014**, *50* (2), 366-78.
23. Ardalan Kholes, S.; Ebrahimi, E.; Jahanzad, E.; Ardalan Kholes, S.; Forghanifard, M. M., MAML1 and TWIST1 co-overexpression promote invasion of head and neck squamous cell carcinoma. *Asia Pac J Clin Oncol* **2018**, *14* (5), e434-e441.
24. Lu, S.; Yu, L.; Mu, Y.; Ma, J.; Tian, J.; Xu, W., et al., Role and mechanism of Twist1 in modulating the chemosensitivity of FaDu cells. *Mol Med Rep* **2014**, *10* (1), 53-60.
25. Li, L.; Wang, Z.; Lu, T.; Li, Y.; Pan, M.; Yu, D., et al., Expression and Functional Relevance of ANXA1 in Hypopharyngeal Carcinoma with Lymph Node Metastasis. *Onco Targets Ther* **2021**, *14*, 1387-1399.
26. Li, Y.; Lu, T.; Hu, G., Gene sequencing and expression of Raf-1 in lymphatic metastasis of hypopharyngeal carcinoma. *Cancer Biomark* **2020**, *28* (2), 181-191.
27. Lamouille, S.; Xu, J.; Derynck, R., Molecular mechanisms of epithelial-mesenchymal transition. *Nature reviews. Molecular cell biology* **2014**, *15* (3), 178-96.

28. Gebauer, F.; Schwarzl, T.; Valcarcel, J.; Hentze, M. W., RNA-binding proteins in human genetic disease. *Nature Reviews Genetics* **2021**, *22* (3), 185-198.
29. Kechavarzi, B.; Janga, S. C., Dissecting the expression landscape of RNA-binding proteins in human cancers. *Genome Biol* **2014**, *15* (1), R14.
30. Zhang, Y.; Li, Z., RNA binding proteins: Linking mechanotransduction and tumor metastasis. *Cancer Lett* **2021**, *496*, 30-40.
31. Zhang, M.; Zhang, Y.; Xu, E.; Mohibi, S.; de Anda, D. M.; Jiang, Y., et al., Rbm24, a target of p53, is necessary for proper expression of p53 and heart development. *Cell Death & Differentiation* **2018**, *25* (6), 1118-1130.
32. Xu, E.; Zhang, J.; Zhang, M.; Jiang, Y.; Cho, S. J.; Chen, X., RNA-binding protein RBM24 regulates p63 expression via mRNA stability. *Mol Cancer Res* **2014**, *12* (3), 359-69.
33. Wycliffe, N. D.; Grover, R. S.; Kim, P. D.; Simental, A., Jr., Hypopharyngeal cancer. *Top Magn Reson Imaging* **2007**, *18* (4), 243-58.
34. Takes, R. P.; Strojan, P.; Silver, C. E.; Bradley, P. J.; Haigentz, M., Jr.; Wolf, G. T., et al., Current trends in initial management of hypopharyngeal cancer: the declining use of open surgery. *Head Neck* **2012**, *34* (2), 270-81.
35. Suzuki, H.; Matoba, T.; Hanai, N.; Nishikawa, D.; Fukuda, Y.; Koide, Y., et al., Lymph node ratio predicts survival in hypopharyngeal cancer with positive lymph node metastasis. *Eur Arch Otorhinolaryngol* **2016**, *273* (12), 4595-4600.
36. Thiery, J. P.; Acloque, H.; Huang, R. Y.; Nieto, M. A., Epithelial-mesenchymal transitions in development and disease. *Cell* **2009**, *139* (5), 871-90.
37. Pastushenko, I.; Blanpain, C., EMT Transition States during Tumor Progression and Metastasis. *Trends Cell Biol* **2019**, *29* (3), 212-226.
38. Morita, Y.; Hata, K.; Nakanishi, M.; Omata, T.; Morita, N.; Yura, Y., et al., Cellular fibronectin 1 promotes VEGF-C expression, lymphangiogenesis and lymph node metastasis associated with human oral squamous cell carcinoma. *Clin Exp Metastasis* **2015**, *32* (7), 739-53.
39. Liu, B.; Li, X.; Li, C.; Xu, R.; Sun, X., miR-25 mediates metastasis and epithelial-mesenchymal-transition in human esophageal squamous cell carcinoma via regulation of E-cadherin signaling. *Bioengineered* **2019**, *10* (1), 679-688.
40. Srivastava, J.; Rho, O.; Youssef, R. M.; DiGiovanni, J., Twist1 regulates keratinocyte proliferation and skin tumor promotion. *Mol Carcinog* **2016**, *55* (5), 941-52.
41. Qin, Q.; Xu, Y.; He, T.; Qin, C.; Xu, J., Normal and disease-related biological functions of Twist1 and underlying molecular mechanisms. *Cell Res* **2012**, *22* (1), 90-106.

Figures

Figure 1

RBM24 was down-regulated in patients with LN metastasis and correlated with poor prognosis in HSCC. a-b Expression of RBM24 in HSCC was confirmed by qRT-PCR and western blot analyses. c Representative IHC staining of RBM24 in HSCC tissues from patients with or without LN metastasis. PBS instead of the primary antibody was used as negative control (left panel, magnification 100x; right panel, magnification 400x). d Kaplan-Meier analysis demonstrated down-regulation of RBM24 was negatively related to overall survival in patients with HSCC (n=57). The upper line represents the survival rate of patients with high expression of RBM24, while the nether line represents the survival rate of patients with low expression of RBM24. *p < 0.05, **p < 0.01

Abbreviations: N, normal tissues; NL, tumour tissues from patients without lymph node metastasis; L, tumour tissues from patients with lymph node metastasis; NC, negative control

Figure 2

Knockdown of RBM24 promoted proliferation, migration and Invasion of FaDu Cells. a-b The mRNA and protein expression levels of RBM24 were down-regulated in RBM24-silenced FaDu cells. c CCK8 cell viability of FaDu cells was detected at 4 different time points. d Colony formation assay was applied to analyze the proliferative ability of FaDu cells. e The migration and invasion capacity of FaDu cells were

determined by transwell assay (magnification 100x). f The motility of FaDu cells was estimated by wound healing assay (magnification 100x). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

Figure 3

Overexpression of RBM24 inhibited proliferation, migration and Invasion of FaDu Cells. a-b The mRNA and protein expression levels of RBM24 were up-regulated in FaDu- OE-RBM24 cells. c CCK8 cell viability

of FaDu cells was detected at 4 different time points. d Colony formation assay was applied to analyze the proliferative ability of FaDu cells. e The migration and invasion capacity of FaDu cells were determined by transwell assay (magnification 100x). f The motility of FaDu cells was estimated by wound healing assay (magnification 100x). *p < 0.05, **p < 0.01, ***p < 0.001

Figure 4

Overexpression of RBM24 down-regulated Twist1 and inhibited EMT of FaDu Cells. a The mRNA levels of Snail, Slug, ZEB1 and Twist1 in FaDu-Control and FaDu-OE- RBM24 cells were determined by qRT-PCR analysis. b The mRNA levels of E-cadherin, N-cadherin and Vimentin in FaDu-Control and FaDu-OE- RBM24 cells by qRT-PCR analysis. c The protein levels of E-cadherin, N-cadherin, Vimentin and Twist1 were examined by western blot analysis. d The expressions of E-cadherin, N-cadherin and Vimentin were measured by confocal immunofluorescence (magnification 400x). **p < 0.01, ****p < 0.0001

Figure 5

Overexpression of Twist1 reversed the inhibiting effects of RBM24 on cell invasion, migration and EMT of FaDu cells. a-b The mRNA and protein expressions of Twist1 in indicated cells. c The migration and invasion capacity of FaDu cells were measured by transwell assay (magnification 100x). d The motility of FaDu cells was detected by wound healing assay (magnification 100x). e The protein expressions of E-

cadherin, N-cadherin, Vimentin and Twist1 in indicated cells. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

Figure 6

Overexpression of RBM24 suppressed HSCC tumour growth and lymph node metastasis in vivo. a Representative image of popliteal lymph node metastasis model. b Representative fluorescence images

of mice injected with FaDu-Control cells and FaDu-OE-RBM24 cells respectively. Yellow arrows indicated footpad tumour, while red arrows indicated popliteal LN. c Image of tumours collected from mice injected with FaDu-Control cells (n=4) and mice injected with FaDu-OE-RBM24 cells(n=5). d The tumour growth curves of the FaDu-Control group (n=4) and the FaDu-OE-RBM24 group(n=5). e Representative images of H&E stained metastatic LNs of the FaDu-Control group (n=4) and the FaDu-OE-RBM24 group (n=5) (left panel, magnification 40x; right panel, magnification 400x). f The ratios of mice with LN metastasis in FaDu-Control group (n=4) and FaDu-OE-RBM24 group(n=5). g Representative IHC staining of tumour cells of FaDu-Control group and FaDu-OE-RBM24 group using anti-RBM24, anti-E-cadherin, anti-Vimentin and anti-Twist1 (magnification 400x). *p<0.05

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryFigure1.tif](#)