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Hsa-miR-337 inhibits non-small cell lung cancer cell invasion and migration by targeting TCF7

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Abstract. – OBJECTIVE: Recent studies have revealed that microRNAs (miRNAs) play a crucial role in the progression of tumorigenesis. Non-small cell lung cancer (NSCLC) is one of the most common malignancies worldwide. The aim of this study was to identify the exact role of hsa-miR-337 in the progression of NSCLC and to investigate the possible underlying mechanism.

PATIENTS AND METHODS: Hsa-miR-337 expression in NSCLC cells and 60 paired tissue samples were detected by Real Time-quantitative Polymerase Chain Reaction (RT-[illegible]. Moreover, the functions of hsa-miR-337 [illegible] were identified by transwell assay and [illegible]d healing assay, respectively. Furthermore, [illegible] underlying mechanism was explored by RT-[illegible]CR, Western blot assay, and luci[illegible]e assay.

RESULTS: The expression le[illegible]miR-337 in NSCLC tissues was rema[illegible]y do[illegible]regulated when compared with t[illegible]f adjac[illegible]normal samples. Moreover, the [illegible]ion a[illegible] of NSCLC cells were [illegible]ign[illegible]t[illegible]nhibite[illegible]ter overexpression [illegible]a-miR[illegible] *in vitro*. Moreover, after overex[illegible]sion of hs[illegible]-337 in vitro, the mRNA [illegible]tein levels [illegible]F7 were significantly [illegible]n-re[illegible]ted. Beside[illegible] the expression of TC[illegible] in NS[illegible]ssues was negatively correlat[illegible]ith the expr[illegible]n of hsa-miR-337.

CONC[illegible]IONS: The abov[illegible]ults suggested that h[illegible]iR-337[illegible]ould repress the migration and inva[illegible] of NS[illegible] cells through directly targeting [illegible]. Fu[illegible]more, [illegible]a-miR-337 might offer a new t[illegible]c inter[illegible]on for NSCLC patients.

Key words:
M[illegible]RNA, Hs[illegible]-337, NSCLC, TCF7.

Introduction

[illegible]g cancer is one of the most common causes of [illegible]cer-related death worldwide, still serving as a threat to public health[1]. As the main type of lung cancer, no[illegible]ll lung canc[illegible]NSCLC) accounts for[illegible]re tha[illegible]% of all lung cancer cases[2]. In the past decades, t[illegible]agnosis and treatment of N[illegible]have been dev[illegible]d greatly. However, t[illegible]ognosis of NSCLC patients remains unsatisfactory. The maj[illegible]y of NSCLC patients are diagnosed in an adva[illegible]d stage, and the 5-year overall s[illegible]al rate of [illegible]ents is less than 15%[3]. Early dete[illegible]ing as the most important step for ther[illegible] strategy, particularly in NSCLC. The reason for this is that there is a lack of clinical [illegible]s at the initial stage. Therefore, it is urgent [illegible]dentify novel biomarkers and therapeutic targets for patients with NSCLC.

MicoRNAs (miRNAs) are a subtype of non-coding RNAs with 18 to 22 nucleotides in length. Several studies have showed that miRNAs play a crucial role in the regulation of many biological behaviors, including cell proliferation, apoptosis, and metastasis in diverse malignancies. For example, activated by K-Ras carcinogenic signal, miRNA-155 facilitates the proliferation of pancreatic cancer cells by regulating reactive oxygen species (ROS) stress[4]. MiR-126 plays an important role in breast cancer by interacting with a variety of molecules. This may eventually help to inhibition of breast cancer cell metastasis[4]. Up-regulation of miR-378 represses the proliferation, migration, and invasion of colon cancer cells by inhibiting epithelial-mesenchymal transition[5]. In addition, miR-532 enhances the invasion of gastric cancer cells *via* activating Wnt/beta-catenin pathway and inhibiting naked cuticle 1 (NKD1). Furthermore, miR-532 may provide a novel therapeutic target for gastric cancer[6]. However, no literature has elucidated the role of hsa-miR-337 in NSCLC metastasis as well as its potential molecular mechanism.

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In our study, we found that hsa-miR-337 was lowly expressed in NSCLC tissue samples. Meanwhile, hsa-miR-337 significantly inhibited NSCLC metastasis *in vitro*. Furthermore, we explored the underlying mechanism of how hsa-miR-337 functioned in NSCLC metastasis.

Patients and Methods

Tissue Specimens

Totally 60 NSCLC patients who underwent surgical resection at The First Affiliated Hospital of Dalian Medical University were enrolled in this study. Human NSCLC tissues and adjacent non-tumor tissues were collected from patients during the surgery. After surgical resection, all tissue samples were snap-frozen in liquid nitrogen immediately for subsequent use. No radiotherapy and chemotherapy treatment were conducted in any patient before the surgery. The study was approved by the Research Ethics Committee of The First Affiliated Hospital of Dalian Medical University. The written informed consents were obtained from all the patients before the study.

Cell Culture and Transfection

Five NSCLC cell lines (A549, SPCA1, H19 H1299, and PC-9) and one i lized h man bronchial epithelial cell (E) were bought from American Ty ulture llection (ATCC; Manassas, VA, All ce re cultured in Dulbecco's M d um (DMEM; Invit n, Ca CA, USA) or Roswell Park orial Inst 1640 (RPMI-1640) medi itrogen, Ca d, CA, USA). Accor g to t nufacturer's protocol of lipofect ine 2000 (I en, Carlsbad, CA, USA), m A mimics (hsa 337; GenePharma, S nai, C ina) was tran ected into cells.

RN tra n and eal Time-c tativ lymerase in Re n -qPCR)

RNA sues and cells was extracted acc ng to the instructions of TRIzol reagent (I lsbad, CA, USA). Complementa- deoxyribose nucleic acids (cDNAs) was syn- sized in strict accordance with Reverse qPCR it (Takara Biotechnology Co., Ltd., Dalian, Ch). SYBR Green Real Time-PCR Master Mix (Roche, Basel, Switzerland) and ABI ViiA7 qPCR System (ABI, Foster City, CA, USA) was used to perform RT-qPCR. The mRNA expression level of hsa-miR-337 was detected. Primers used in this study were as follows: hsa-miR-337, forward 5'-CGCTTCAGCTCCTATATGA-3' a 5'-GTGCAGGGTCCGAGGT-3'; U forwa 5′-CTCGCTTCGGCAGCACA-3′ reverse 5′-AACGCTTCACGAATTTGC TCF7, forward 5′-CTCGAGCCTA AAGT-GACA-3′ and reverse TTTA AG GCTTTGAAAAACAA 3′; Glycera 3-phosphate dehydroge (GAP , forwa TGTGGGCATCAA TTT 3′ and everse 5′-ACACCATGTA AAT-3′ ecific thermal cycle w 0 sec C, 5 at 95°C for 40 cycles 35 sec at 60

Wound ealing ay

Six-well plates we d to culture NSCLC cel miRNA mim re transfected into c When growing to about 90% of confluence, s were scrat by a sterile 10 μL pipette tip. , the cells w incubated in a humidified in- c r with 5% O_2 atmosphere at 37°C. Open wou easured at 24 h. These proce- dures w ated for three times.

ell Assays

24 fter transfection, 2×10^5 cells in 100 μL of serum-free DMEM were seeded into the upper chamber of 8-μm culture inserts (Corning, Corning, NY, USA). Then, 20% of fetal bovine serum (FBS)-DMEM (Invitrogen, Carlsbad, CA, USA) was added to the lower chamber of culture inserts. After culturing for 24 h, the cells were fixed with methanol for 30 min and stained with hematoxylin for 20 min. Finally, migrated cells were observed under an inverted microscope (×20), and the number of cells was calculated. Five fields were randomly selected for each sample.

Western Blotting Analysis

48 h after transfection, cells were first collected. Radioimmunoprecipitation assay (RIPA) protein extraction reagent (Beyotime, Shanghai, China) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland) and phenylmethylsulfonyl fluoride (Roche, Basel, Switzerland) was used to lyse cells. Subsequently, total proteins were separated by 10% Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred onto 0.22-μm NC membranes. The membranes were incubated with specific primary and secondary antibodies. An-

ti-GAPDH and anti-TCF7 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Luciferase Reporter Gene Assay

The 3′-Untranslated Region (3′-UTR) of TCF7 was cloned into a pGL3 vector as wild-type (WT) 3′-UTR. Site-directed mutagenesis of hsa-miR-337 binding site in TCF7 3′-UTR was used as mutant (MUT) 3′-UTR by quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Then, the plasmids were transfected into A549 cells for 48 h. Dual luciferase reporter assay system was used for performing luciferase reporter gene assay.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 (SPSS, Chicago, IL, USA) statistical software was used for all statistical analysis. The independent-sample *t*-test was used to compare the difference between the two groups. *p*-values<0.05 were considered statistically significant.

Results

Hsa-MiR-337 Was Lowly Expressed in NSCLC Tissue Specimens a[illegible] Lines

First, RT-qPCR was cond[illegible] ect hsa-miR-337 expression in 60 [illegible]ents' [illegible]ues and 4 NSCLC cell lines. R[illegible] show[illegible] miR-337 was significa[illegible]ly [illegible] mor tissues than th[illegible] adjac[illegible]sues (Figure 1A). Compared w[illegible]bHBE cells, [illegible]xpression of hsa-miR-337 [illegible]C cells was [illegible]ficantly lower (Figure [illegible]).

Overexpression of Hsa-MiR-337 Inhibited Migration and Invasion of NSCLC Cells

According to hsa-miR-337 expression in NSCLC cells, A549 cells were chosen fo[illegible] pression of hsa-miR-337. Hsa-miR[illegible] mimi[illegible] and negative control (NC) were s[illegible]etized and transfected into A549 cells. Su[illegible]ntly, hsa-miR-337 expression was ve[illegible]ified [illegible] qPCR (Figure 2A). Wound heal[illegible] assay [illegible] that overexpression of hsa-mi[illegible]7 inhibited [illegible] cell migration (Figure[illegible]. More[illegible], the res[illegible]s of the transwell assa[illegible] onst[illegible] that th[illegible] over-expression of hsa[illegible]R-[illegible]icantly [illegible]bited the invasion of [illegible]LC ce[illegible]ure[illegible].

Hsa-MiR-[illegible]bited Tum[illegible]genesis of NSCLC [illegible] Targ[illegible] TCF7

Starbase v2.0 onli[illegible]tabase predicted that TC[illegible] a possible tar[illegible]f hsa-miR-337 (Figu[illegible]A). Luciferase reporter gene assay showed significantly[illegible]s luciferase activity was ob-[illegible]d in A549 [illegible]LC cells co-transfected with h[illegible]R-337 mi[illegible] and WT-TCF7-3′-UTR when com[illegible]ntrol group (Figure 3B). Results of West[illegible] assay showed that the protein ex-[illegible]ression of TCF7 was remarkably down-regulated [illegible] miR-337 mimics transfection (Figure 3C).

Correlation Between Expression of TCF7 and Hsa-MiR-337

RT-qPCR results further demonstrated that the mRNA expression of TCF7 was significantly up-regulated in NSCLC cells when compared with 16HBE cells (Figure 4A). Meanwhile, TCF7 expression in NSCLC tissues increased markedly when compared with that of adjacent tissues (Figure 4B). Furthermore, the results of linear correlation analysis indicated that in NSCLC tissues,

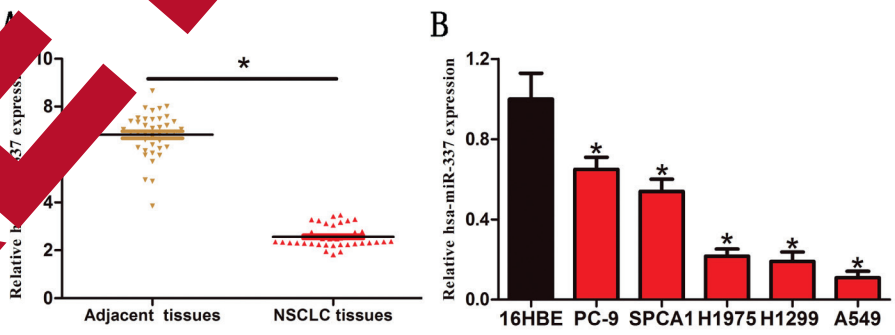

Figure 1. Expression level of hsa-miR-337 was significantly decreased in NSCLC tissues and cell lines. ***A,*** Hsa-miR-337 expression was significantly decreased in NSCLC tissues when compared with adjacent normal tissues. ***B,*** Expression level of hsa-miR-337 in human NSCLC cell lines and normal gastric epithelial cell line (A549) was determined by RT-qPCR. Data were presented as mean ± standard error of the mean. *$p<0.05$.

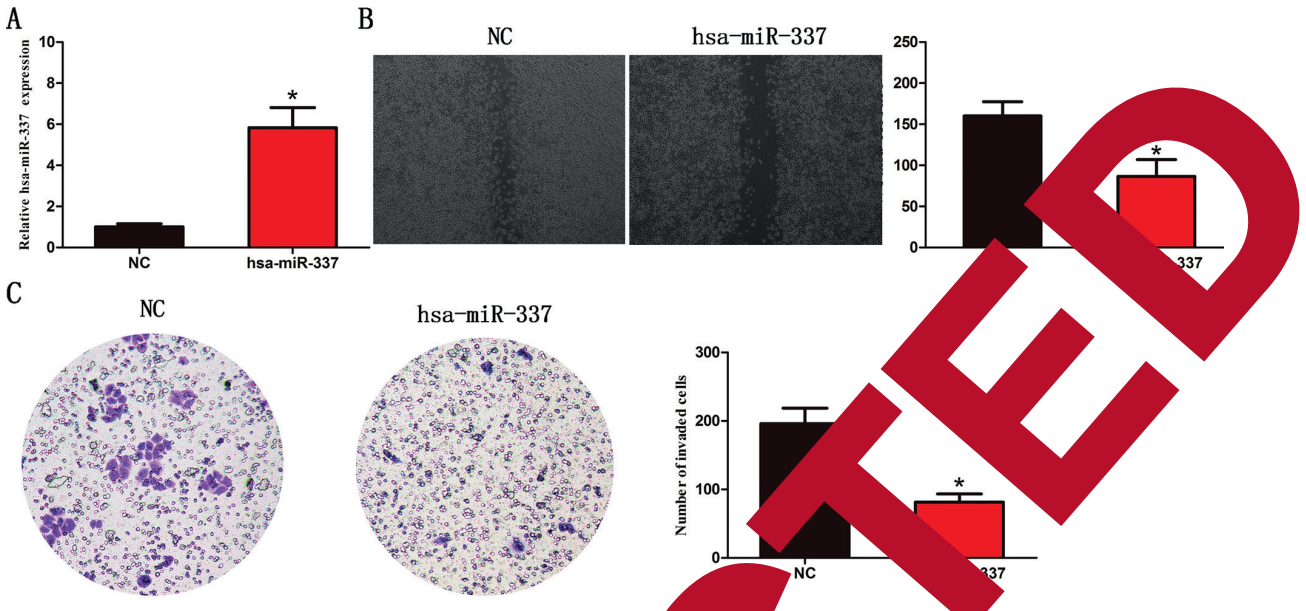

Figure 2. Overexpression of hsa-miR-337 inhibited migration and invasion of A549 NSCLC cells. ***A,*** Hsa-miR-337 expression in cells transfected with negative control (NC) or hsa-miR-337 mimics (hsa-miR-337) was detected by RT-qPCR. ***B,*** Wound healing assay showed that the overexpression of hsa-miR-337 significantly suppressed the migration ability of hsa-miR-337 cells compared with NC cells. ***C,*** Transwell assay demonstrated that the number of invaded cells was significantly decreased in hsa-miR-337 transfected cells compared with NC cells. Results represented the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$, as compared with NC cells.

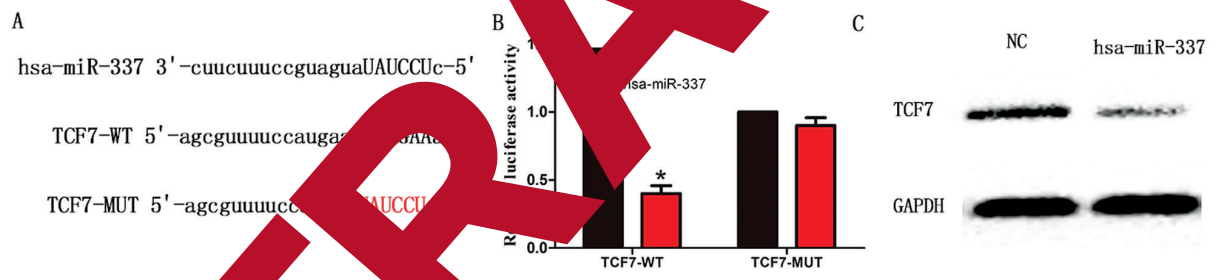

Figure 3. Hsa-miR-337 repressed NSCLC cells metastasis process via inhibiting TCF7. ***A,*** The binding sites of hsa-miR-337 on TCF7. ***B,*** Luciferase reporter gene assay showed that cells transfected with hsa-miR-337 mimics exhibited less luciferase activity than those transfected with NC. ***C,*** Hsa-miR-337 mimics repressed the protein expression of TCF7 in A549 NSCLC cells. *$p<0.0$

the expression of TCF7 was negatively correlated with miR-337 expression (Figure 4C).

Discussion

Dysregulation of miRNA activity leads to changes in homeostasis and various pathological process. Researches have proved that miRNAs exhibit important regulatory functions in carcinogenesis of NSCLC. For example, Zhao B. et al[8] have shown that over-expression of lncRNA PCAT-1 promotes the proliferation and metastasis of NSCLC. Xue et al[9] have reported that through down-regulating SOCS1, SOCS6, and PTEN, miR-21 and miR-155 promote the progression of NSCLC. Meanwhile, this is inversely correlated with the prognosis of NSCLC patients. In addition, Zhao L. et al[10] have found that miR-149 suppresses the growth and metastasis of NSCLC cells *via* depression of FOXM1/cyclin D1/MMP2 axis. Moreover, Wang et al[11] have proved that miR-142-5p inhibits the proliferation of NSCLC through targeting PIK3CA.

MiR-337 is reported to play an important role in the progression of diverse carcinomas. For ex-

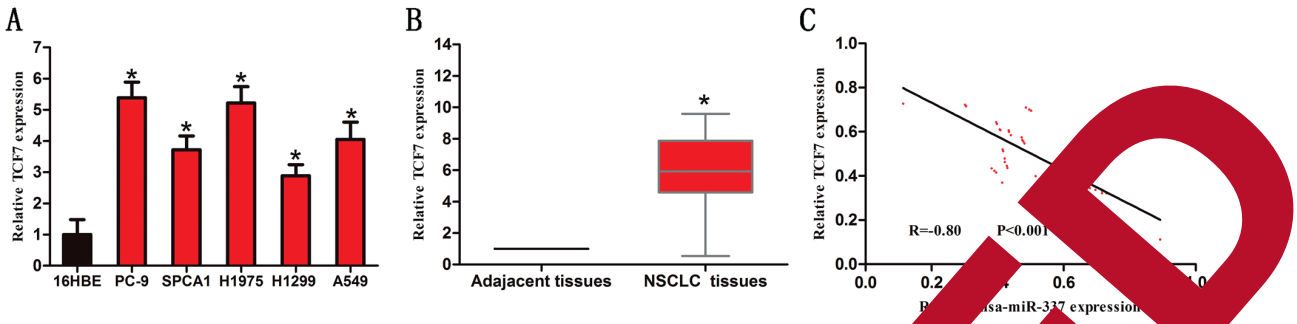

Figure 4. TCF7 expression in NSCLC tissues and NSCLC cell lines. ***A,*** TCF7 was high[illegible]ressed i[illegible]CLC cell l[illegible]s compared with 16HBE cells. ***B,*** TCF7 expression in NSCLC tissues was significantly [illegible]than [illegible]of corre[illegible]onding non-tumor tissues. ***C,*** Correlation analysis revealed that hsa-miR-337 expression was [illegible]gat[illegible]ated wit[illegible]7 expression in NSCLC tissues. *$p<0.05$.

ample, miR-337 inhibits the development of melanoma by targeting STAT3. This indicates that miR-337 may serve as a potential therapeutic target for the melanoma[12]. MiR-337, functioning as an oncogene, promotes the proliferation of endometrial carcinoma by depressing PTEN expression[13]. MiR-337 acts as a tumor suppressor in the progression of colorectal cancer through tar[illegible]g KRAS directly and repressing the AKT a[illegible] pathways[14]. Moreover, miR-337 exerts a su[illegible]s-sion effect on pancreatic ductal adenocarci[illegible] through regulating cell proliferation and invas[illegible] by targeting HOXB7[15]. In this w[illegible]a-miR-33[illegible] was found significantly dow[illegible]in both NSCLC tissues and cell[illegible]urther[illegible]e, after hsa-miR-337 overexpres[illegible]the abi[illegible]f cell migration and invasio[illegible] w[illegible] pressed. These data[illegible]icated[illegible]hsa-miR-337 functioned as a t[illegible] suppresso[illegible] inhibited the tumorigene[illegible]CLC.

Transcripti[illegible]actor[illegible](TCF7) plays an important ro[illegible]promoti[illegible]progression of diverse ca[illegible]s. For instance[illegible]ough activation of W[illegible]signali[illegible] and up-regulation of TCF7, mi[illegible]34a r[illegible]sses bone metastasis in prostate[illegible]r[illegible]ated h[illegible]Ras[16]. The overexpression of[illegible]92 sup[illegible]ses the proliferation and [illegible]otes [illegible]o[illegible]s of human osteosarcoma by[illegible]eting[illegible]. MiR-6852 functions as an ant[illegible]cogene in colorectal cancer. Meanwhile, m[illegible]presses tumor progression and [illegible]astasis through targeting TCF7, which may [illegible]ve as a potential prognostic marker and ther-[illegible]c target for colorectal cancer[18]. In addition, by[illegible]ding to TCF7 in Wnt signaling pathway, AF1q enhances cell proliferation, migration, and chemoresistance in breast cancer[19]. In our study, results of RT-qPCR a[illegible]stern blot analysis indic[illegible]t TCF7 was [illegible]icantly down-regula[illegible]after hsa-miR-337 overexpression *in vitro*. [illegible]at's more, TC[illegible]was remarkably up-regulated [illegible]SCLC samp[illegible]when compared with that of a[illegible]nt tissues[illegible]egative correlation was found bet[illegible]ssion of TCF7 and hsa-miR-337 in NCS[illegible]es. The above results revealed that [illegible]sa-miR-337 might realize its function *via* target-[illegible]F7.

Conclusions

Our research indicates that hsa-miR-337 plays a vital role in the carcinogenesis of NSCLC, which can serve as a promising biomarker for NSCLC.

Conflict of interest

The authors declare no conflicts of interest.

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