MicroRNA let-7c inhibits migration and invasion of human non-small cell lung cancer by targeting ITGB3 and MAP4K3

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ABSTRACT

MicroRNAs play an important regulatory role in carcinogenesis and cancer metastasis. Different members of let-7 family have been reported to be decreased in human lung tumors. However, the effect of specific let-7 member on metastasis of NSCLC remains undefined. Our current study detected the expression of let-7 members in 94 cases of NSCLC and a significant association was noticed between low levels of let-7c expression and metastasis, venous invasion, advanced TNM stages and poor survival of NSCLC patients. Consistently, ectopic expression of let-7c in relatively highly metastatic cells remarkably suppressed their migration and invasion. Inhibition of let-7c in cells with relatively low metastatic potential promoted their motility and invasion. We then analyzed the potential targets of let-7c and found that ITGB3 and MAP4K3 were directly repressed by let-7c. Upon restoring the expression of ITGB3 and MAP4K3, the effects of let-7c on tumor metastasis were partially reversed, and more importantly, the expression levels of ITGB3 and MAP4K3 were inversely correlated with let-7c in 64 NSCLC tissues. Collectively, our results suggest that let-7c, by degrading ITGB3 and MAP4K3, prevents NSCLC metastasis.

1. Introduction

Lung cancer is the leading cause of cancer mortality worldwide, with nearly 1,400,000 deaths each year [1]. Non-small cell lung cancer (NSCLC) accounts for almost 85% of lung cancer. About 40% stage I and 60% stage II NSCLC patients are dying of distant metastases within 5 years after curative tumor resection [2]. Thus, identification of new molecules involved in tumor metastasis is a critical step toward the development of novel therapeutics. These new agents are expected to prevent distal metastasis, thereby significantly increasing the survival rate.

MicroRNAs (miRNAs) are recognized as important post-transcriptional regulators of gene expression [3]. Mounting evidence suggests that the aberration or alteration of miRNAs is involved in carcinogenesis, progression and metastasis in many human cancers including NSCLC [4]. Yanaihara and colleagues, by comparing 104 NSCLC samples to corresponding normal lung tissues, have demonstrated that 43 miRNAs are differentially expressed in cancer samples [5]. High levels of mir-328 and mir-378 are found to be associated with brain metastasis from NSCLC [6,7]. Low level of mir-451 expression is reportedly associated with lymph node involvement of NSCLC [8]. Recently, meta-analysis of prospective trials reveals that mir-21 and mir-155 are reliable predictors for recurrence, metastasis and poor survival of NSCLC patients [9,10]. Thus, the potential role of miRNAs on NSCLC metastasis warrants further investigation.

Let-7 family has been shown to act as tumor suppressors by inhibiting oncogenes and key regulators of mitogenic pathways including RAS, MYC, HMGA2, etc. [11]. As for lung cancer, some let-7 members are reported to be correlated with tumor growth and pathologic classification. Let-7a and let-7f are firstly reported to be down-regulated in human lung cancers and are proved to be correlated with poor prognosis [12]. Ectopic overexpression levels of let-7b and let-7g are able to effectively suppress cancer growth in mouse models of lung cancer [13–15]. Expression profiles of let-7 members are also a promising method for classification of adenocarcinoma (AD) and squamous cell carcinoma (SCC) [16]. These studies suggest that specific member of let-7 family plays a crucial role in the growth and development of lung cancer. However, the influence of let-7 members on metastasis in lung cancer remains largely undefined. Our previous findings suggest that let-7c is a suppressor in migration and invasion of colorectal cancer (CRC) [17]. Our current study aimed to generate more results consolidating the correlation between expression of let-7
members (let-7a, 7b, 7c) and metastasis of human lung cancer cells. We focused on let-7c because its expression was significantly down-regulated both in the lung cancer cell lines with relatively highly metastatic potential and in tumor tissues with metastasis. The effect of let-7c on migration and invasion of lung cancer cells was directly verified by cell functional assays after overexpression and inhibition of let-7c in cells. To explore the potential mechanism underlying let-7c mediated metastatic suppression in lung cancer cells, a list of candidate genes were predicted by bioinformatics' software and further confirmed by dual-luciferase reporter system. As a result, both ITGB3 and MAP4K3 are identified as target genes responsible for the functions of let-7c. ITGB3 (integrin β3, also known as CD61), a member of integrin family, has been shown to be involved in cell adhesion and "outside-in" signaling transduction [18]. MAP4K3, a member of the MAPK4 family, is an important mediator in different signaling pathways [19]. Our study first demonstrates that let-7c, in addition to its suppressive role in tumor growth, also inhibits tumor metastasis by directly destabilizing the mRNAs of ITGB3 and MAP4K3 in NSCLC.

2. Materials and methods

2.1. Cell lines and clinical samples

Human NSCLC cell lines (SKMES-1, H520, H157, A549, GLC82, H1299) and human lung epithelium cell (HLEC) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Grand Island, NY) in a humidified atmosphere of 5% CO2 at 37 °C incubator.

The NSCLC specimens (n = 94) without any preoperative radiotherapy or chemotherapy and matched adjacent normal tissues obtained from the patients undergoing surgery at Beijing Cancer Hospital during 2006–2008 with informed consent following the protocols approved by the Ethics Committee of Peking University Hospital.

2.2. RNA extraction and quantitative RT-PCR

Total RNA was extracted from tissues and cells according to the protocol of miRNeasy Mini Kit (Qagen, Valencia, CA, USA). For mature miRNAs quantification, 100 ng total RNA was added with a polyA tail by polyA polymerase (New England Biolabs, Beverly, MA, USA), following by reverse transcription with an oligo-dT adapter primers and Moloney murine leukemia virus (MMLV, Invitrogen). For mRNA detection, cDNAs were synthesized from 2 μg total RNA using oligo-d(T)15 primers and MMLV. Sequences of all primers were listed in Table S1. qRT-PCR was performed by using SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) on LightCycler® 480 Real-Time PCR System (Roche, USA). The relative amount of miRNAs or genes was normalized to U6 or GAPDH. Data was calculated based on 2^DCT where ΔCT = CT (Target) – CT (Reference). Fold change was calculated by the 2^-ΔΔCT method.

2.3. Plasmid construction and cell transfection

pcDNA3.0-pre-Let-7c was constructed in our previous study [17] and transfection into SKMES-1 cells by lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Stable single let-7c overexpressing cell clones were screened by G418 (500 μg/ml) and then expanded culture. Pre-Let-7c was also subcloned into plenti6-TREpitt vectors to construct tetracycline-inducible let-7c expression system. Shuttle vector constructs and the ViraPower Packaging Mix (Invitrogen) were cotransfected 293FT cells according to the manufacturer's protocol to obtain lentivirus. Lentivirus was then added to SKMES-1 cells and screened by Blasticidin (5 μg/ml) and G418 (500 μg/ml) to generate TET-ON let-7c expression cells.

To rescue expression of MAP4K3, the open reading frame (ORF) of MAP4K3 was amplified from cDNA library by PCR and cloned into the lentiviral shuttle vector plenti6 (Invitrogen). The lentiviral plasmid containing ITGB3 was obtained from GeneCopoeia (EX-E2219-LV105).

2.4. Cell proliferation assay

To observe the effect of let-7c on cell proliferation, SKMES-1 cells with tetracycline-inducible let-7c expression were seeded into 96-well plates and induced by doxycycline (Dox, 2 μg/ml). Cell Counting Kit-8 (CCK-8) was used to monitor cell growth at 0, 1, 2, 3, 4 day and the number of viable cells was assessed by measurement of absorbance at 450 nm by FLUOstar OPTIMA (BMG LAB-TECH, Offenburg, Germany).

2.5. Wound healing assay

About 3 x 10^5 cells were seeded in 6-well dishes and an incision was made in the central area of the confluent culture to create an artificial wound. Images of the wound area were captured by microscope (Leica, Wetzlar, Germany) 24 h after injury.

2.6. Transwell cell migration and invasion assay

To evaluate the effect of let-7c on cell migration and invasion, 2.5 x 10^4 cells in 100 μl RPMI1640 with 1% FBS were plated to the upper chamber of the transwells (8 μm pore size, Corning, NY, USA) with and without matrigel. The lower chamber was loaded with 450 μl of RPMI1640 with 10% FBS as chemoattractants for cells. After 24 h, migrated or invaded cells were counted and imaged by microscope after fixing with 2% methanol and stained with 1% crystal violet solution.

2.7. Bioinformatic prediction and dual-luciferase reporter assay

miRGen was used to analyze the putative target genes of let-7c. The 3’-UTRs of ITGB3 and MAP4K3 containing predicted let-7c binding sites were amplified from cDNA library. The corresponding mutant 3’-UTRs were obtained by overlap-extension PCR. The sequences of all primers were listed in Table S1. All PCR products were cloned into downstream of firefly luciferase gene of the pGL3-control plasmid (Promega, Madison, WI). SKMES-1 cells were cotransfected with 200 ng of pGL3 constructions, 600 ng of pcDNA3.0 or pcDNA3.0-let-7c and 26 ng of pRL-TK in 24-well plates by Lipofectamine 2000. Luciferase activity (Firefly and Renilla) was measured with a dual-luciferase reporter assay system (Promega) at 24 h after transfection.

2.8. Protein extraction and western blot analysis

Protein was extracted from cells using RIPA buffer containing complete protease inhibitor cocktail (Roche, Mannheim, Germany). Protein (20 μg) was separated by electrophoresis on SDS–PAGE gels and blotted onto the PVDF membrane (Millipore). Rabbit anti-ITGB3 (1:1000 dilution, Epitomics, USA); rabbit anti-MAP4K (1:1000 dilution, Cell Signaling Technology, USA); mouse anti-β-actin (1:50,000 dilution, Roche) were used as primary antibodies. HRP-conjugated goat anti-rabbit or anti-mouse IgG (cwBiotech, China) was used as secondary antibody respectively. Signals were visualized by using chemiluminescence (Millipore).
2.9. Overexpression or inhibition of let-7c by miRNA mimics or inhibitors

The synthetic let-7c mimics (5′-UGAGGUAGGUAGGUUAGGGU-UU-3′) and negative control miRNA (5′-CAGUACUUUUGUUGUACGUACGUAA-3′) were synthesized by GenePharma (Shanghai, China). A chemically-modified let-7c-inhibitor (5′-AACAUACACUACUACUACUACGUUCU-3′) and negative control (5′-UUGUGUACUACACAAAGUACUG-3′) were purchased from RiboBio (Guangzhou, China). Cells were transiently transfected with oligonucleotides using lipofectamine 2000 and were used to evaluate migratory and invasive behavior. At the same time, cells were also collected to examine the expression of let-7c, ITGB3 and MAP4K3.

2.10. Statistical analysis

Continuous variables with normal distribution were presented as mean ± SD, otherwise were presented as median. Dichotomous variables were presented as number and percentage values. Differences between each group were assessed by two-tailed Students’ t-test or Mann–Whitney test by SPSS13.0 unless specified. A statistical significance was found among different pathologic types, for example, let-7c expression was higher in adenocarcinoma (AD) than in squamous cell carcinoma (SCC). Although there seemed an inverse correlation between let-7c expression and advanced node stage, statistical analysis did not reveal a significant association. In contrast, there were no significant differences in let-7c expression with regards to the genders, ages and T grades.

3. Results

3.1. Let-7c down-regulated in human lung cancer cells with highly metastatic ability

The invasive potential of lung cancer cell lines used in this study was firstly tested by transwell assay as described in Supplementary Fig. 1. The expression levels of let-7c in these cell lines were detected to determine the potential effect of let-7c expression on metastasis. Fig. 1A showed that the relative expression level of let-7c was lower in cells with relatively highly metastatic ability (H1299, SKMES-1 and A549) than those with relatively low or no metastatic potential (H157, H520, GLC82, HLEC). These results suggested that low level of let-7c might be associated with NSCLC metastasis.

3.2. Decreased let-7c expression associated with metastasis in NSCLC patients

To determine the relationship between let-7c and metastasis in clinical samples, we firstly examined let-7c expression in 38 human NSCLC tissues and matched normal tissues. As shown in Fig. 1B, let-7c expression was significantly down-regulated in NSCLC tissues compared to normal tissues. Because we focused on the role of let-7c expression in tumor metastasis, we further examined let-7c expression in 94 NSCLC samples. As a result, low level expression of let-7c was associated with venous invasion, advanced TNM stage and lymph node or distant metastasis. As listed in Table 1, the median value of relative let-7c expression in tissues with venous invasion was about 50% lower than in tissues without venous invasion (p = 0.0219), 38% lower in tissues with advanced TNM stage than with TNM I-II stage (p = 0.0357) and 45% lower in tissues with metastasis than those without metastasis (p = 0.0098). A statistical significance was found among different pathologic types, for example, let-7c expression was higher in adenocarcinoma (AD) than in squamous cell carcinoma (SCC). Although there seemed an inverse correlation between let-7c expression and advanced node stage, statistical analysis did not reveal a significant association. In contrast, there were no significant differences in let-7c expression with regards to the genders, ages and T grades.

3.3. Decreased let-7c expression associated with poor survival of NSCLC patients

Kaplan–Meier curves were used to analyze survival of patients with NSCLC categorized by a cut-off value according to median
expression level of let-7c (0.725). As shown in Fig. 1C and D, tumor free survival (TFS) and overall survival (OS) time with low let-7c expression were significantly shorter than those with high let-7c expression. The 75 percentile of TFS for low versus high let-7c expression group were 11.43 versus 42.88 months ($p$ = 0.0342), and the 75 percentile of OS for the low versus high let-7c expression group were 25 versus 41 months ($p$ = 0.0386). Taken together, low level of let-7c expression may predict a short survival time of NSCLC. However, COX regression failed to demonstrate the expression of let-7c as an independent prognostic factor (data not shown).

3.4. Let-7c inhibited growth, migration and invasion of SKMES-1 cells in vitro

The relationship of reduced expression of let-7c and metastasis of NSCLC drove us to explore the possible biological functions of let-7c in lung cancer cells. We first induced let-7c expression in the highly metastatic SKMES-1 cells. As shown in Fig. 2A, the relative expression of let-7c peaked 48 h after adding 2 μg/ml DOX. Overexpression of let-7c in SKMES-1 cells showed distinct patterns of growth, spreading and invasion inhibition compared with control cells (Fig. 2B–F). To improve the result, we further transiently transfected the other two highly metastatic cell lines H1299 and A549 with let-7c mimics. After transfected with mimics, let-7c expression in H1299 and A549 cells were strongly increased compared with control cells (Supplementary Fig. 2). As shown in Fig. 2G and H, cells migrated through transwell were reduced by 74% and 77% in transfected H1299 and A549 respectively, compared with control cells. Moreover, invasive cells were also decreased by 69% and 88% in transfected H1299 and A549 respectively, compared with control cells. Consistent with data from SKMES-1 cells, overexpression of let-7c in both cells also showed a reduced cell migration and invasion by transwell assay. Collectively, overexpression of let-7c may impede cellular proliferation, cell migration and invasion in vitro.

3.5. Let-7c directly down-regulated ITGB3 and MAP4K3 expression by binding to their 3'-UTRs

To investigate the mechanism through which let-7c overexpression suppresses tumor growth and metastasis, we firstly predicted the target genes of let-7c by miRGen and screened out 28 candidate genes associated with cell growth and invasion. To identify the genuine targets, the changes of these genes were detected in SKMES-1 cells with induced let-7c expression. Among these genes, the expression levels of MAP4K3 and ITGB3 were significantly repressed after let-7c overexpression both in mRNA and protein levels compared to control cells (Fig. 3A and B). The binding sites in 3'-UTRs of ITGB3 and MAP4K3 for let-7c and the corresponding mutated sequences were depicted in Fig. 3B. To determine whether the regulation is direct, the 3'-UTRs of ITGB3 and MAP4K3 containing let-7c binding-sites and corresponding mutations were cloned into the pGL3-control vector. As shown in Fig. 3D, the relative luciferase activity was significantly decreased in the wild-type 3'-UTRs of ITGB3 and MAP4K3.

Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>Case number (%)</th>
<th>Let-7c expression ($2^{-ΔΔCt}$)</th>
<th>$p$ value$^1$</th>
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<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>65 (69.1)</td>
<td>0.6832</td>
<td>0.7997</td>
</tr>
<tr>
<td>Female</td>
<td>29 (30.9)</td>
<td>0.8129</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;62 yr</td>
<td>50 (53.2)</td>
<td>0.6529</td>
<td>0.9486</td>
</tr>
<tr>
<td>&gt;62 yr</td>
<td>44 (46.8)</td>
<td>0.8343</td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>11 (11.7)</td>
<td>1.501</td>
<td>0.1628</td>
</tr>
<tr>
<td>Moderate</td>
<td>41 (43.6)</td>
<td>0.5819</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>39 (41.5)</td>
<td>0.6977</td>
<td></td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>3 (3.2)</td>
<td>0.4607</td>
<td></td>
</tr>
<tr>
<td>Histological type</td>
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<td></td>
<td></td>
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<tr>
<td>Adenocarcinoma</td>
<td>58 (61.7)</td>
<td>0.9498</td>
<td>0.0139</td>
</tr>
<tr>
<td>Squamous carcinoma</td>
<td>30 (31.9)</td>
<td>0.5074</td>
<td></td>
</tr>
<tr>
<td>Adenosquamous carcinoma</td>
<td>3 (3.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large-cell carcinoma</td>
<td>3 (3.2)</td>
<td></td>
<td></td>
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<tr>
<td>Venous invasion</td>
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<td></td>
<td></td>
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<tr>
<td>Yes</td>
<td>15 (16.5)</td>
<td>0.4402</td>
<td>0.0219</td>
</tr>
<tr>
<td>No</td>
<td>79 (83.5)</td>
<td>0.8617</td>
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<tr>
<td>Tumor stage</td>
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</tr>
<tr>
<td>T1</td>
<td>21 (22.3)</td>
<td>0.8548</td>
<td>0.0918</td>
</tr>
<tr>
<td>T2</td>
<td>53 (56.4)</td>
<td>0.6811</td>
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<tr>
<td>T3</td>
<td>16 (17.0)</td>
<td>0.7541</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>4 (4.3)</td>
<td>0.3883</td>
<td></td>
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<td>Node stage</td>
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<td></td>
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<tr>
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<td>40 (42.6)</td>
<td>0.9794</td>
<td>0.1947</td>
</tr>
<tr>
<td>N1</td>
<td>28 (29.8)</td>
<td>0.774</td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>26 (27.7)</td>
<td>0.5422</td>
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</tr>
<tr>
<td>TNM stage</td>
<td></td>
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<tr>
<td>I-II</td>
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<tr>
<td>III</td>
<td>39 (41.5)</td>
<td>0.5353</td>
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<tr>
<td>Metastasis</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>32 (34)</td>
<td>0.5422</td>
<td>0.0098</td>
</tr>
<tr>
<td>No</td>
<td>62 (66)</td>
<td>0.8848</td>
<td></td>
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</tbody>
</table>

$^1$ Mann-Whitney test was used to evaluate the significant associations for two groups and Kruskal–Wallis test for more groups.
compared to corresponding mutant constructs and the control. These data revealed that let-7c directly decreased the expression of these genes through binding to their 3'-UTRs.

To confirm the correlation of let-7c and target genes in lung cancer tissues, we examined the expression of let-7c and the mRNA levels of ITGB3 and MAP4K3 in the same set of 64 NSCLC tissues. As expected in Fig. 4, let-7c expression was inversely correlated with the mRNA expression levels of two target genes. The results supported that let-7c targeted ITGB3 and MAP4K3 in vivo.

3.6. Inhibition of let-7c enhanced cell migration and invasion in GLC82 cells by increasing the expression of ITGB3 and MAP4K3

To further confirm the results, we inhibited the expression of let-7c by miRNA inhibitors in relatively low metastatic cell lines, including GLC82, H520 and H157 cells. The levels of let-7c expression were decreased about 50% after transfected with let-7c inhibitor compared with control cells (Fig. 5A). Consistent with the results of overexpressing let-7c in SKMES-1 cells, the inhibition of let-7c expression in GLC82, H157 and H520 cells enhanced the cell motility and invasion (Fig. 5B and C). At the same time, the protein levels of ITGB3 and MAP4K3 were up-regulated after inhibition of let-7c in GLC82 cells compared with negative control (Fig. 5D). These data supported that inhibition of let-7c expression increased cell motility and invasion through terminating the degradation of its target genes ITGB3 and MAP4K3.

3.7. Rescue of ITGB3 and MAP4K3 reversed the effect of let-7c on metastasis

To investigate whether ITGB3 and MAP4K3 have an important role in let-7c-mediated inhibition of metastasis, we rescued the expression of ITGB3 and MAP4K3 in let-7c overexpressing SKMES-1 cells (Fig. 6A and B). The inhibitory effect of let-7c on cell migration and invasion was overcome after rescue of ITGB3 and MAP4K3 (Fig. 6C–E). Collectively, these results suggested that let-7c acted as a metastatic suppressor in lung cancer cells by repressing the expression of ITGB3, MAP4K3, and possibly other molecules.

4. Discussion

In this study we firstly determine the expression level of let-7 members in lung cancer cell lines and clinical samples. The decreased expression of let-7c is found to be associated with highly invasive ability in lung cancer cells and metastasis in NSCLC. Then we investigate the mechanism underlying the let-7c-mediated suppression of metastasis. As a result, ITGB3 and MAP4K3 are identified as direct target genes of let-7c. After rescuing expression of ITGB3 and MAP4K3, the suppressive effects of let-7c on migration and invasion of NSCLC cells are partially reversed. Taken together, our work suggests that let-7c exerts its suppressive effect on metastasis of NSCLC by targeting ITGB3 and MAP4K3.
Our previous study has demonstrated that let-7c is a metastatic suppressor in colorectal cancer [17]. It is also reported that let-7c expression is significantly decreased in metastatic prostate cancer compared to high grade but localized carcinoma [20]. Here, we demonstrate that let-7c also plays a suppressive role in migration and invasion of NSCLC cells. In addition, we confirm that reduced let-7c expression is associated with venous invasion, advanced TNM stage, and poor survival of NSCLC. Furthermore, Fassina and

![Figure 3](image1.png)

**Fig. 3.** Let-7c targeted ITGB3 and MAP4K3 directly in SKMES-1 cells. (A and B) The relative expression levels of MAP4K3 and ITGB3 both in mRNA and protein levels were suppressed in SKMES-1 cells after inducing expression of let-7c. (C) Putative let-7c binding sites for let-7c and corresponding mutations in 3'-UTRs of ITGB3 and MAP4K3. (D) Luciferase reporter assay demonstrated that let-7c inhibited the wild-type, but not the mutant of 3'-UTRs of ITGB3 and MAP4K3 reporter activities compared with the vector control ($p < 0.05$). Data represented the mean ± SD of three independent experiments with quadruplicates of each sample.

![Figure 4](image2.png)

**Fig. 4.** The correlation analysis suggested significantly negative correlation between let-7c expression and MAP4K3 and ITGB3 mRNA levels in 64 NSCLC tissues. $p$ values were obtained by two-tailed Spearman’s test.
Fig. 5. Inhibition of let-7c up-regulated the ITGB3 and MAP4K3 expression and elevated the migration and invasion in GLC82 cell line. (A) The expression level of let-7c was decreased about 50% by let-7c inhibitor. (B and C) Cell migration and invasion were increased after inhibition of let-7c in GLC82, H520 and H157 cells. Each bar represented the mean ± SD of the counts from three respectively experiments. Bar: 50 μm. (D) The protein levels of ITGB3 and MAP4K3 were increased after inhibition of let-7c compared to the control cells.

Fig. 6. Rescue of ITGB3 and MAP4K3 alone overcame the effects of let-7c on migration and invasion in let-7c overexpressing SKMES-1 cells. (A and B) Expression levels of ITGB3 and MAP4K3 were increased both in mRNA and protein levels after introducing two genes in let-7c overexpressing SKMES-1 cells. (C) Cell migration (upper) and invasion (lower) abilities were partially reversed after introducing two genes in let-7c overexpressing SKMES-1 cells. Bar: 50 μm. (D and E) Data represented the mean ± SD of three independent experiments with four random fields counted for each chamber. *p < 0.001.
colleagues have shown that let-7c expression is higher in AD than that in SCC [16], which has been also identified in our study. Collectively, the observations made by other groups and our own suggest that let-7c, in addition to its role in proliferation [21], immunocyte differentiation [22], drug resistance [23] and carcinogenesis [24], is closely involved in tumor metastasis. Bcl-xl, HMGA2 and MYC have been identified as the let-7c targets in different human cancers [25–27]. In the present study, we also investigate the mechanism responsible for the let-7c-mediated inhibition of NSCLC metastasis and first demonstrate that ITGB3 and MAP4K3 are two target genes of let-7c as mediators for NSCLC migration and invasion. ITGB3 (integrin β3), a molecule involved in several diseases including cancer [28,29], can promote ROS-induced migration and invasion in colorectal cancer cells [30]. It works as a downstream molecule of let-7a to incite development and metastasis of malignant melanoma [31]. ITGB3, as a target gene of mir-30, is up-regulated in breast tumor-initiating cells, leading to an anti-apoptotic effect [32]. Our results indicate that ITGB3 is a direct target of let-7c. Accordingly, rescuing ITGB3 expression can reverse the inhibitory effects of let-7c on migration and invasion in NSCLC. Furthermore, we reveal an inverse correlation between let-7c expression and ITGB3 expression in 64 NSCLC tissues. Together with the observations that let-7a inhibits metastasis of malignant melanoma by increasing ITGB3 expression [31], it is plausible for us to postulate that different let-7 members may target same gene for regulating similar biological function in different cancer cells. MAP4K3 (GLK) is a member of MAP4K family containing a conserved N-terminal kinase domain, C-terminal citron homology domain and proline-rich motifs in center [19]. Previous data have demonstrated that MAP4K3 is connected with JNK [19], apoptosis [33], EGFR [34] and mTOR [35,36] signaling pathways. Our own findings suggest that MAP4K3 is a target gene of let-7c and restoration of MAP4K3 expression abolishes the inhibitory effect of let-7c on cell migration and invasion. In addition, let-7c expression is inversely correlated with expression levels of MAP4K3 in NSCLC tissues. Thus, our results suggest that ITGB3 and MAP4K3 are let-7c target genes involved in NSCLC progression, which may help us to identify new therapeutic targets for lung cancer.

Interestingly, our previous study has shown that the let-7c target genes also include MMP11 and PBX3, both of which are involved in the let-7c-mediated suppression of migration and invasion in CRC [17]. To determine whether there is a signaling pathway through ITGB3 or MAP4K3 to induce expression of MMP-11, we find links between ITGB3 or MAP4K3 and MMP-11 by the BioGraph (http://biograph.be/) as shown in Attachment 1. Results from BioGraph prediction suggest that the potential pathways for increasing expression of MMP11 through ITGB3 and/or MAP4K3 are so complicated. As for the emerging data suggests female sex hormones play a key role in stimulating NSCLC progression [37], whether ITGB3 induces MMP11 expression through estradiol and progesterone pathway is still worthy of further study. Given that the inhibitory effect of tumor metastasis is mediated by different target genes, further studies are warranted to establish either a linear signal cascade or interactive crosstalk among ITGB3, MAP4K3, MMP11 and/or PBX3.

To summarize, our study indicates that let-7c plays a pivotal role in progression and metastasis of NSCLC by down-regulating its target genes ITGB3 and MAP4K3. These findings lend novel insight to the mechanism underlying NSCLC metastasis and might facilitate the development of prognostic biomarkers as well as effective treatment regimens.

Conflict of Interest statement

None declared.

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Appendix A. Supplementary material

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References


