Original Article MiR-205 is implicated in migration and invasion of non-small cell lung cancer H460 cells through targeting vimentin

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Abstract: Lung cancer is one of the most common malignancies and the leading cause of cancer related death in the world. Despite of advances in several treatment strategies, the prognosis of advanced non-small cell lung cancer (NSCLC) is unfavorable. MicroRNAs have been implicated in cancer development and progression. In this study, we investigated the role of miR-205 in the migration and invasion of NSCLC. To generate tumor-repopulating cells (TRCs), we cultured NSCLC cell line H460 in three-dimension (3D) soft fibrin gels. H460-TRCs cultured in 3D soft fibrin gels exhibited increased migration and invasion ability, along with significantly up-regulated expression of EMT and stemness associated genes and protein. Moreover, further investigation on the microRNAs profile of H460-TRCs showed that the expression of miR-205 was decreased in H460-TRCs. Down regulating the expression of miR-205 with inhibitor in H460 led to enhanced migration and invasion ability and increased Vimentin protein expression. We focused on miR-205 and identified its target according to bioinformatics analysis. Further data showed that silencing Vimentin resulted in decreased colony-formation, migration and invasion ability. These results suggested that miR-205 may play a critical role through vimentin in NSCLC migration and invasion.

Keywords: miR-205, migration, invasion, NSCLC, Vimentin

Introduction

Lung cancer is one of the most common malignancies and the leading cause of cancer-related death in the world [1]. According to histopathology, lung cancer can be classified into two broad subtypes including non-small cell lung cancer (NSCLC), which is the cause of 85% of cases, and small cell lung cancer (SCLC), which accounts for 15% of cases [2, 3]. Despite of a variety of treatment strategies for lung cancer, the 5-year survival rate of localized or metastatic advanced NSCLC is still unfavorable [4]. Distant metastasis of primary tumor is the major cause of tumor progression and recurrence [5, 6].

Lately, accumulating studies have identified cancer stem cells (CSC) as a unique subpopulation of cancer cells [7, 8]. These cells possess self-renewal and differentiation abilities as well as enhanced proliferation, migration and invasion properties. Tumor growth, metastasis and recurrence are closely related with CSC. Therefore, investigation on NSCLC CSC would provide new sight into the underlying mechanism of tumor metastasis. Currently, conventional methods used for the identification, isolation and cultivation of CSC depending on CSC surface markers are often unreliable and lack specificity [9, 10]. A mechanical method to generate CSC from general tumor cell lines through culturing tumor cells in 3D soft fibrin gels was newly developed. Cells selected in these methods are functionally named as TRCs because of their novel migration and invasion activity and tumorigenesis in mice [11, 12]. Selecting TRCs by using 3D soft fibrin gels culture will facilitate the investigation on CSC and the mechanism underlying tumor migration and invasion.

Metastasis is a complex process involving multiple steps, including tumor growth, angiogenesis, EMT, intravascular delivery, cellular anchoration and so on. In recent years, studies have carried out to investigate genes and their products that drive or regulate metastasis process. Studies have reported that EMT played a critical role in tumor development, progression, invasion, metastasis and stemness. EMT is a process in which epithelial cells lose polarity. intercellular adhesion decreases and cellular movement is elevated and the cellular polarity and junction are consequently destroyed. In this process, the expression of junctional protein and epithelial specific protein (e.g., E-cadherin, Claudin-1, ZO-1) decreases, while the expression of mesenchymal specific protein (e.g., Vimentin, N-cadherin) increases [13]. Vimentin is a kind of intermediate filament associated protein encoded by gene vimentin, which regulates the interaction between cytoskeletal protein, adhesion protein and other protein. Researches have indicated that Vimentin is a specific marker of EMT, and is involved in adhesion to tumor related epithelial cells and macrophages, migration, invasion and cellular signal transduction of cancer cells [14].

In addition to alterations in protein-encoding genes, abnormalities in non-coding genes also contribute to cancer pathogenesis [15, 16]. MicroRNAs are a class of small non-coding RNAs that participate in post-transcriptional regulation of genes expression. Although microRNAs generally identify target genes by completely or incompletely pairing with 3'-untranslated region in mRNA, they can also regulate target genes expression by combining 5'-untranslated region or coding sequences in mRNA. Increasing evidence indicated that microRNAs were implicated in the regulation of a variety of cellular processes, including proliferation, apoptosis, metastasis and invasion of tumor and cancer stem cells (CSC), thus contributed to tumor initiation, development and regression [17]. To date, miRNAs can function as either oncogenes or tumor suppressors. Over 60 abnormally expressed microRNAs have been identified in NSCLC [18-20]. In NSCLC, microRNAs interact with target genes to affect the proliferation, EMT, chemoradiotherapy sensitivity and resistance of tumor. For instance, let-7 and miR-451 were negatively related to the risk and prognosis of NSCLC respectively. In

addition, upregulation of miR-126 could suppress adhesion, invasion and metastasis of lung cancer. It has also been reported that miR-145 inhibited NSCLC proliferation, and miR-34 induced tumor apoptosis and blocked cell cycle of tumor cells [21, 22]. Recently, we found that miR-205 was significantly down-regulated in NSCLC H460-TRCs. However, some previous data from other groups showed high expression of miR205 in both lung cancer tissues and cell lines [23, 24]. Besides, its function on tumor initiation, migration and invasion is controversial. miR205 may promote NSCLC proliferation, migration and invasion by inhibiting PTEN [25]. On the other hand, it could suppress tumor migration in NSCLC cell line via down-regulating low density lipoprotein receptor related protein [1, 26].

Although abnormal expression of miR-205 in NSCLC has been reported, it is debated whether miR-205 acts as an oncogene or tumor suppressor [27, 28]. Moreover, the precise parts played by miR-205 in specific steps of NSCLC progression, especially metastasis, are still unknown. For these reasons, we undertook to investigate the potential role of miR-205 in NSCLC cell metastasis. By selecting H460-TRCs using 3D soft fibrin gels culture, here we assessed the expression of miR-205 in TRCs, and examined its effects on tumor migration. invasion and the expression of EMT-related genes. Furthermore, we explored the target of miR-205 in H460. Taken together, our study might provide insights into the causal mechanisms of NSCLC invasion and metastasis.

Materials and methods

Cell lines and cell culture

NSCLC cell line H460 was purchased from the Shanghai Institute of Cell Biology (Shanghai, China). Cells were propagated in RPMI-1640 (Gibco), and the medium was supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin. Cells were cultured at 37° C in 5% CO₂.

3D soft fibrin gels culture

3D soft fibrin gels were prepared as previously described [11]. Firstly, salmon fibrinogen (Pfenex, San Diego, USA) and T7 buffer were mixed in a ratio of 1:9. Then H460 single cell suspension was added to equivolume fibrino-



Figure 1. H460-TRCs cultured in 3D soft fibrin gels possessed enhanced migration and invasion ability. A. Morphology, number and area of H460-TRCs when cultured in 3D soft fibrin gels. B. Analysis of the migration and invasion in H460 cultured in traditional 2 dimension plates (2D) and H460-TRCs generated in 3 dimension soft gels (3D). C. CCK-8 analysis of proliferation in H460 (2D) and H460-TRCs (3D). Values are the mean \pm SE of three replicates. ***, *P* < 0.001 compared with the control.

gen-T7 mixture. Next, 250 µl mixture solution above was blended with 5 µl thrombin as soon as the thrombin (Pfenex, San Diego, USA) was added to the precooled 24-wells plate. The plate was placed in incubator for 20 min to make sure the stabilization of 3D soft fibrin gels. Finally, 1 ml complete medium was added slowly into the plate and cells were cultured as mentioned above. H460-TRCs were collected after incubation for 3 to 5 days. Following removal of the culture medium, the remaining 3D soft fibrin gels were mechanically destroyed and dissolved in 5 × dispase II solution in incubator for 15 min and were shaken every 3 min. After centrifugation of the dissolved gels, H460-TRCs were collected and washed with PBS. H460-TRCs were then digested with trypsin for 5 min and washed with PBS to separate

clonal spheres into single cells. Cells were finally suspended in complete medium for further experiments.

Cell migration and invasion assay

In vitro cell migration assay was conducted using 24-wells migration chambers with 8.0 μ m pores (Corning, USA). For cell invasion assay, chambers should be precoated with 50 to 70 μ l Matrigel (BD Biosciences, Bedford, MA). Cells were seeded at 50000 to 100000 cells in 200 μ l serum free medium in upper chamber. Then 500 μ l complete medium containing 10% FBS was added to the lower chamber. After incubated for 24 h, cells were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet solution for 20 to 40 min. Chambers



Figure 2. Several EMT and stemness associated genes were abnormally expressed in H460-TRCs. A. mRNA expression of EMT associated genes including β -catenin and N-cadherin, slug, snail, twist, vimentin, zeb1 in H460 (2D) and H460-TRCs (3D). B. Relative mRNA expression of stemness associated genes including nanog and sox-2 in H460 (2D) and H460-TRCs (3D). C. Western bot analysis of EMT and stemness associated genes. Values are the mean ± SE of three replicates. *, P < 0.05, **, P < 0.01, ***, P < 0.001 compared with the control.

were washed and wiped with cotton swab to remove cells on the top surface. Cells attached to the bottom surface were photographed and quantified under a light microscopy (× 200). Each group was assayed in duplicate. The average number of cells in 10 fields chosen at random in each chamber was calculated.

Cell proliferation assay

Cell proliferation assay was performed using CCK-8 (Dojindo, Japan). Cells were seeded in 96-wells plate at 5000 cells/well. After incubation overnight, cells were treated with or without cis-platinum for 24 h or 48 h. Next, 10 µl CCK-8 was added to the medium for further 2 h incubation. Finally, the absorbance at 450 nm was measured with a microtiter plate reader (Bio-Rad, CA) and calculated as cell proliferation rate and inhibit rate of cis-platinum.

RT-PCR and real time PCR

Total RNA of H460 and H460-TRCs were extracted with TRIzol reagent (Invitrogen) for

RT-PCR and real time PCR. cDNA were synthesized from 2 µg total RNA using reverse transcription system (Promega). Real time PCR was conducted with FastStart Universal SYBR Green Master Kit (Roche) on an ABI 7900 System. Small endogenous nucleolar U6 siRNA and GAPDH were used as control for normalization of miRNA and mRNA expression respectively. The primers for siRNA were from Gene-Pharm, Shanghai, China and the primers for mRNA were provided by TSINGKE, Wuhan, China.

Western blot analysis

Cells were collected and lysed with NP40 supplemented with Protease inhibitor Cocktail. After separation by SDS-PAGE, proteins were transferred to PVDF membrane, and blocked for 2 hours in TBS containing Tween 20 and 5% BSA. Subsequently, the membranes were incubated with primary anti-bodies and followed by incubation with horseradish peroxidase (HRP)conjugated secondary antibodies. Anti- β -catenin, anti-N-cadherin, anti-slug, anti-snail, anti-



Figure 3. H460-TRCs exhibited different microRNAs expression profiles compared with H460. miR-141 and miR-205 were significantly down-regulated in H460-TRCs (3D) compared with H460 (2D). Values are the mean \pm SE of three replicates. **, *P* < 0.01 compared with the control.

Twist, anti-Vimentin, anti-ZEB1, anti-Claudin, anti-SOX-2, anti- β -actin (Cell Signaling, Danvers, MA) were used according to the manufacturer's instructions. The antibody-protein complexes were detected by using ECL detection system and photographed with the ChemiDoc XRS system (Bio-Rad, USA).

Transfection

Cells were seeded in 6-wells plate and incubated overnight. When cells were 70% to 90% confluent, they were transfected with siRNAs of vimentin, miR205 inhibitor or negative control (GenePharm, Shanghai, China) using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The silence efficacy of siRNAs and inhibitor was confirmed using Real-time PCR.

Statistical analysis

Data were shown as mean values + SEM. Statistical analysis was conducted using the SPSS15.0 software. Differences were statistically significant when the p value was less than 0.5. Differences between groups were compared using the Student's t test.

Results

Biological characteristics of H460-TRCs cultured in 3D soft fibrin gels

To select H460-TRCs, we cultured H460 in 3D soft gels and observed the morphology and clonal growth of tumor cells for 6 days. The data showed that most of the tumor cells died and only part of the tumor cells grew into clonal

spheres. The number of clonal spheres declined while the volume increased, especially from the third day of culture (Figure 1A). To further determine the migration and invasion ability of H460-TRCs, we conducted Transwell assay. Compared with H460 cultured in traditional 2 dimension plates (2D), H460-TRCs generated by 3D soft gels (3D) migrated and invaded much more significantly (Figure 1B). Next, we examined the proliferation of H460-TRCs using CCK-8 assay. The result showed a little higher proliferation activity of H460-TRCs than H460 but there was no significant difference between the two groups (Figure 1C). These results demonstrated that H460-TRCs cultured in 3D soft fibrin gels possessed enhanced migration and invasion ability.

EMT and stemness associated gene and protein profile of H460-TRCs

To further investigate the mechanism underlying the enhanced migration and invasion ability of H460-TRCs, we explored the EMT and stemness associated gene expression profile of H460-TRCs using Real-time PCR. We identified 5 EMT associated genes expressed at higher level in H460-TRCs, including slug, snail, twist, vimentin, zeb1, while the expressions of β-catenin and N-cadherin were only slightly different between H460-TRCs and H460 (Figure 2A). Besides, we found that sox-2, one of the most important stemness associated genes, was highly expressed in H460-TRCs (Figure 2B). Western blot analysis helped us to determine whether the protein expressions of those genes were also up-regulated. The Western blot analysis showed that the expressions of EMT associated protein including Vimentin, ZEB-1, β-catenin, Snail, N-cadherin and stemness associated protein SOX-2 were higher in H460-TRCs compared with H460. In contrary, Claudin-1 was down-regulated in H460-TRCs (Figure 2C). The results illustrated the abnormal expression of several genes were related with EMT and stemness of cancer cells.

Different microRNAs profile of H460-TRCs and H460

To identify the microRNAs potentially involved in H460-TRCs migration and invasion, we examined microRNAs expression between H460-TRCs and H460 through Real-time PCR. The data showed that several microRNAs exhibited different expression levels in the two groups. In



Figure 4. Inhibition of miR-205 promoted the migration and invasion ability and up-regulated vimentin expression in H460. A. The efficacy of miR-205 inhibitor in H460. B. Transwell assays of H460 after transfection with miR-205 inhibitor. C. Bioinformatics analysis of potential target for miR-205. D. mRNA and protein expression of vimentin in H460 after transfection with miR-205 inhibitor. Values are the mean \pm SE of three replicates. **, *P* < 0.01, ***, *P* < 0.001 compared with the control.

particular, miR-141 and miR-205 were significantly down-regulated in H460-TRCs (**Figure 3**). Among them, miR-205 was focused on as it was more closely related with tumor migration and invasion according to references.

The role of inhibited miR-205 in promoting migration and invasion ability and up-regulating Vimentin in H460

To illustrate the function of miR-205 on H460 migration and invasion, we down-regulated miR-205 by miR-205 inhibitor and conducted Transwell assay. We tested the transfection efficiency of miR205 inhibitor and data showed that miR-205 was down-regulated as much as 68% by miR-205 inhibitor (**Figure 4A**). Significantly increased migration and invasion rate was observed in H460 treated with miR-205 inhibitor (**Figure 4B**).

We next utilized bioinformatics software to analyze the potential target genes of miR-205. Data from Target Scan suggested 4413 genes including 624 conserved and 5350 less conserved binding sites as possible target genes for miR-205. In view of the association with tumor migration and invasion and increased expression in H460-TRCs as previously proved, we prioritized to analyze the complementary sequences in zeb-1, vimentin and miR-205 (**Figure 4C**). zeb-1 has already been identified as target gene of miR-205, however, whether it is regulated by miR205 is still unclear.

In order to investigate the function of miR-205 on vimentin, we down-regulated miR-205 through transfection with miR-205 inhibitor. Next, we determined the expression of vimen-



Figure 5. Inhibition of vimentin by siRNA inhibited the migration and invasion ability and colony formation of H460. A. The efficacy of siRNA1270 and siRNA1746 in H460. B. Transwell analysis of H460 after transfection with siRNA1270 and siRNA1746. C. Colony formation of H460 in 3D soft fibrin gels after transfection with siRNA1270 and siRNA1746. Values are the mean \pm SE of three replicates. **, *P* < 0.01, ***, *P* < 0.001 compared with the control.

tin through Real-time PCR and western blot analysis after transfection with miR-205 inhibitor or negative control. We found that the expression of vimentin was obviously up-regulated after inhibition of miR-205, while there was no significant difference in gene expression (Figure 4D).

These results illustrated that inhibition of miR-205 enhanced the migration and invasion ability in H460. Meanwhile, down-regulated miR-205 was associated with increased expression of vimentin in H460.

Influence of down-regulated vimentin on the migration and invasion of H460 and the clonal sphere formation in 3D soft fibrin gels

In order to find out whether vimentin could contribute to migration and invasion of H460, we transfected siRNA targeting vimentin and conducted Transwell assay. We tested the transfection efficiency and found that the inhibition rate of siRNA1270 or siRNA1746 was about 50% (Figure 5A). The inhibition activity was also confirmed through western blot. Compared with H460 treated with negative control, the migration and invasion activity of H460 was much lower in siRNA1270 and siRNA1746 groups (Figure 5B). We next set out to observe whether vimentin could affect the clonal sphere formation when H460 were cultured in 3D soft fibrin gels for 6 days. Data showed that fewer tumor cells grown into clonal spheres and their clonal area and volume were much smaller in siRNA1270 and siRNA1746 group (Figure 5C). These results demonstrated that inhibition of vimentin impaired both the migration and invasion ability and the clonal sphere formation of H460.

Discussion

In our study, H460-TRCs cultured in 3D soft fibrin gels were proved to possess novel cancer stem cell like properties, especially higher migration and invasion activity. Besides, we found that the expression of miR-205 was decreased in H460-TRCs. Further investigations testified that inhibited miR-205 was involved in up-regulating the expression of vimentin to promote migration and invasion in NSCLC H460. These results identified vimentin as a new target for miR-205 and provided insight into the function and mechanism of miR205 in regulating migration and invasion in lung cancer.

CSC is an important factor involved in tumor migration and invasion. Methods based on stem cell surface markers and other conventional methods have been used in CSC isolation and cultivation. However, these methods are often unstable, unreliable and cannot provide enough cells for further investigation

as the isolated cells cannot largely proliferate in vitro [9, 10]. In our study, we used 3D soft fibrin gels to select and grow H460-TRCs from general tumor cells. 3D soft fibrin gels culture is a newly established method based on biomechanics material to isolate and culture CSC. Stem cells are softer than differentiated cells. When they are cultured in 3D soft fibrin gels made of soft, noncytotoxic and nonimmunogenic salmon fibrin-matrigel, CSC survive and proliferate while differentiated tumor cells undergo apoptosis as they cannot accommodate to soft environment [12, 29]. Cells isolated and cultured in 3D soft fibrin gels are defined as TRCs according to their high tumorigenesis in animal models and migration and invasion ability [11, 12]. Our results proved that H460-TRCs had higher migration and invasion ability, which is consistent with previous research. However, there was no significant difference in proliferation activity. It is a probable reason that TRCs quickly turn into differentiated tumor cells when they return to 2-dimension environment in CCK-8 assay.

Our study found that miR-205 was down-regulated in H460-TRCs and inhibited miR-205 promoted H460 migration and invasion. MiR-205 may function as tumor suppressor or promoter in different tumors and target to different genes. miR-205 was highly expressed in lung cancer reported in several studies [23, 30, 31]. Zarogoulidis P and colleagues declared that miR-205 was highly expressed in A549 and H1975, and resulted in resistance to cis-platinum by down-regulating pro-apoptosis protein and up-regulating anti-apoptosis protein [24]. In contrast, data from Larzabal et al showed that high expression of miR-205 participated in up-regulating the expression of E-cadherin and down-regulating the expression of ZEB1, thus further inhibited tumor growth, migration and adhesion [32].

In view of these present studies, miR-205 is closely related with NSCLC, but its function on NSCLC development and progression is worthy of further exploration. Consistent with the latter study, our investigation on H460 indicated negative correlation between miR-205 expression and the ability of migration and invasion. Besides, we also verified vimentin as a new target for miR-205 in regulating tumor migration and invasion. In our study, inhibition of miR-205 resulted in up-regulation of vimentin and enhanced tumor migration and invasion. Down-regulation of vimentin inhibited migration and invasion ability of H460 and clonal sphere formation in 3D soft fibrin gels. However, whether miR-205 regulates vimentin directly or indirectly is not investigated in our work. Therefore, luciferase reporter assay is needed to determine whether vimentin was a direct target for miR-205.

In conclusion, our results indicated that miR-205 was down-regulated in H460-TRCs, and its down-regulation promoted migration and invasion of H460. This biologic effect of miR-205 may be due to modulated expression of vimentin.

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Disclosure of conflict of interest

None.

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