MicroRNA-29b attenuates non-small cell lung cancer metastasis by targeting matrix metalloproteinase 2 and PTEN

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Abstract

Background: Our pilot study using miRNA PCR array found that miRNA-29b (miR-29b) is differentially expressed in primary cultured CD133-positive A549 cells compared with CD133-negative A549 cells.

Methods: Ten human non-small cell lung cancer (NSCLC) cell lines and samples from thirty patients with NSCLC were analyzed for the expression of miR-29b by quantitative RT-PCR. Bioinformatics analysis combined with tumor metastasis PCR array showed the potential target genes for miR-29b. miR-29b lentivirus and inhibitors were transfected into NSCLC cells to investigate its role on regulating cell proliferation which was measured by CCK-8 assay in vitro and nude mice xenograft tumor assay in vivo. Cell motility ability was evaluated by transwell assay. The target genes of miR-29b were determined by luciferase assay, quantitative RT-PCR and western blot.

Results: Bioinformatics analysis combined with tumor metastasis PCR array showed that matrix metalloproteinase 2 (MMP2) and PTEN could be important target genes of miR-29b. The expression of miR-29b was down regulated in NSCLC tissues compared to the normal tissues. Clinicopathological analysis demonstrated that miR-29b had significant negative correlation with lymphatic metastasis. The gain-of-function studies revealed that ectopic expression of miR-29b decreased cell proliferation, migration and invasion abilities of NSCLC cells. In contrasts, loss-of-function studies showed that inhibition of miR-29b promoted cell proliferation, migration and invasion of NSCLC cells in vitro. Nude mice xenograft tumor assay confirmed that miR-29b inhibited lung cancer growth in vivo. High-invasion (A549-H) and low-invasion (A549-L) NSCLC cell sublines from A549 cells were created by using the repeated transwell assay aimed to confirm the effect of miR-29b on migration and invasion of NSCLC. Furthermore, the dual-luciferase reporter assay demonstrated that miR-29b inhibited the expression of the luciferase gene containing the 3'-UTRs of MMP2 and PTEN mRNA. Western blotting and quantitative RT-PCR indicated that miR-29b down-regulated the expression of MMP2 at the protein and mRNA levels.

Conclusion: Taken together, our results demonstrate that miR-29b serves as a tumor metastasis suppressor, which suppresses NSCLC cell metastasis by directly inhibiting MMP2 expression. The results show that miR-29b may be a novel therapeutic candidate target to slow NSCLC metastasis.

Keywords: miR-29b, NSCLC, Metastasis, MMP2, PTEN
Background
Lung cancer is characterized by a low survival and high relapse rate after surgery [1, 2]. NSCLC, the most frequently occurring category of lung cancer, accounts for approximately 80% of all cases [3]. Tumor invasion and metastasis are the main factors responsible for NSCLC treatment failure [4, 5].

Emerging evidence has revealed that microRNAs (miRNAs) play key roles in various biological processes, including metastasis, proliferation, apoptosis, stress resistance, tumorigenesis, and cell differentiation [6, 7]. Compared with normal tissues, different tumors have distinct miRNA expression characteristics. It has been determined that abnormal miRNA expression is a critical carcinogenesis signal. In tumors, miRNAs perform functions similar to those of oncogenes and tumor suppressors. miRNA expression patterns are more finely regulated than those of proteins [8]. Given these findings, miRNA may have great potential as a biomarker in early tumor diagnosis and treatment targets [9]. Many studies have attempted to identify the metastasis-related miRNAs in metastatic tumors using miRNA microarrays analysis. For example, several miRNAs have been identified to be involved in development of NSCLC metastasis, including let-7 [10], miR-200 [11], miR-125b [12] and miR-10b [13]. Although miRNAs have been the subject of extensive research in recent years, the molecular regulatory mechanisms of miRNAs and their effects on cancer are not well understood.

In the present study, to screen the metastasis-related miRNAs of NSCLC, CD133-positive and CD133-negative subpopulation from human lung adenocarcinoma A549 cells were isolated through immunomagnetic bead separation method. CD133 has been considered a specific stem cell marker and NSCLC prognosis marker [14, 15]. CD133-positive cells have greater potential for proliferation, metastasis, and chemo-radioresistance [16–18]. Through bioinformatics analysis and miRNA PCR array and tumor metastasis PCR array, PTEN, ETV4, COL4A2 and MMP2 were logically been speculated as miR-29b target genes. Our results showed that miR-29b inhibited growth and metastasis of NSCLC cells in vitro and vivo. Additionally, dual-luciferase reporter assay and western blot results further elucidated that the miR-29b inhibited the expression of the luciferase gene containing the 3'-UTRs of MMP2 and PTEN mRNA. While miR-29b down-regulated the expression of MMP2 at the protein and mRNA levels. The results showed that miR-29b maybe a novel therapeutic candidate target or strategy for seeking to control NSCLC metastasis.

Methods
Tissue samples, cell culture and animals
Information about tissue specimens, NSCLC cell lines and animals is given in the Additional file 1.

Microarray screening of differentially expressed genes between CD133-positive/negative NSCLC cells
Detailed information about isolation of CD133-positive/negative A549 cells and microarray screening of differentially expressed genes is provided in the Additional file 1.

Bioinformatics analysis
Using “miRNA” as the index word for predicting target genes in the TargetScan (www.targetscan.org), PicTar (www.picтар.org), and miRanda (www.microrna.org) databases, target genes were identified from overlapping results from the three databases. Subsequently, we extracted the overlap of these results with that of the tumor metastasis PCR array.

Quantitative RT-PCR
Quantitative RT-PCR was performed using kits for U6 and mature miR-29b (ABI, Foster City, CA, USA), according to the manufacturer's instructions. SYBR green real-time RT-PCR was performed to detect MMP2 and PTEN. Detailed information is provided in the Additional file 1.

Transfection studies
All miRNA duplexes (Additional file 2: Table S1) were purchased from Genepharma (Shanghai, P.R. China). H460 cells were transfected with inhibitor or inhibitor NC at a final concentration of 100 nmol/L using Lipofectamine RNAiMAX instructions (Invitrogen). A549 subline stably expressing miR-29b (A549-miR-29b) and its control line (A549-NC) were established as described in the Additional file 1.

Western blotting
The cells were lysed with radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China). The antibodies used for western blotting are described in the Additional file 1.

Plasmid construction
We purchased psCHECK-2 plasmids from Promega (Madison, WI, USA). Human genome DNA was used as the template for the MMP2 3’ untranslated region (UTR) and PTEN 3’ UTR PCR. XhoI and NotI restriction sites were introduced at the 5’ ends of both the forward and reverse primers (Additional file 2: Table S1). Following double digestion, the linear psCHECK-2 fragment was connected with the 3’ UTRs using T4 DNA ligase; positive clones were selected for sequencing validation after transformation. The sequencing-validated, target recombinant plasmid was designated psCHECK-2-Wt-MMP2/PTEN-3’ UTR, and was used as a template for constructing psCHECK-2-Mut-MMP2/PTEN-3’ UTR using antisense PCR and a site-specific mutagenesis kit (Toyobo, Osaka, Japan).
Luciferase reporter activity assay
Plasmid (0.5 μg) and 50 nmol/L miR-29b mimic/mimic NC were cotransfected using Lipofectamine LTX reagent (Invitrogen). Three replicates and three parallel lines were used each time. Following 48-h transfection, luciferase activity was measured using a GloMax 20/20 Luminometer (Promega) according to the dual luciferase reporting system instructions. Relative luciferase activity was compared using the ratio of Renilla reniformis and firefly luciferase activity (Rn/Ff).

Cell proliferation assay
To measure the effect of miRNA and inhibitor on cellular proliferation rates, cells were incubated in 10 % CCK-8 (DOJINDO) diluted in normal culture media at 37 °C until visual color conversion appears. Proliferation rates were determined at 24, 48, 72, 96, 120 h post-transfection, and quantification was done on a microplate reader set according to the manufacturer’s protocol.

In vitro assays of migration and invasion
The 24-well Boyden chamber with 8-μm pore size polycarbonate membrane (Corning, NY) was used to analyze the migration and invasion of tumor cells. Details are in the Additional file 1.

In vivo studies
H460 subline stably knockdown miR-29b (H460-LV-miR-29b inhibitor) and its control line (H460-LV-CON), were established as described in Additional file 1. Analysis for tumorigenicity was performed as described in Additional file 1.

Statistical analysis
All data were analyzed using SPSS 13.0 (SPSS Inc, Chicago, IL, USA); A paired t test was used to investigate the difference in the expression level of miR-29b between normal and cancerous tissues. A 2-sample t test was used to analyse the clinicopathologic characteristics of miR-29b expression in the tissues of patients with NSCLC. Quantitative RT-PCR, CCK-8 assay, migration and invasion assay, and luciferase reporter assay were tested using 1-way analysis of variance for factorial design. P value < 0.05 was considered statistically significant.

Results
Screening and identifying the metastasis-related miRNAs and target genes of NSCLC
To explore the miRNAs related to NSCLC metastasis, miRNA PCR array (MAH-3100A detected 376 human disease–related miRNA) were used to evaluate miRNA expression in primary cultured CD133-positive/negative A549 cells. Fourteen miRNAs were found up-regulated and thirty-seven miRNAs were down-regulated in CD133-positive cells (Fig. 1a, Additional file 3: Table S2). The human tumor metastasis PCR array (PAHS-028A detected 84 metastasis-related genes) was used to further determine the metastasis-related genes that could be controlled by CD133-regulated miRNAs. Nineteen metastasis-related genes were found up-regulated in CD133-positive cells (Fig. 1b, Additional file 4: Table S3). Finally, the target genes of significantly different miRNAs were predicted by bioinformatics analysis. The overlap genes were found between bioinformatics predicted analysis and tumor metastasis PCR array. Among the predicted target genes of the seven down-regulated miRNAs in CD133-positive A549 cells, the tumor metastasis PCR array contained four target genes of miR-29b (Fig. 1c). MiR-29b was down-regulated 7.6-fold in CD133-positive cells. However, its putative target genes PTEN, ETV4, COL4A2, and MMP2 were up-regulated 1.11 to 4.2-fold. Based on PTEN and MMP2 were reported closely related to metastasis process, these two genes were further investigated to confirm their regulation by miR-29b in NSCLC.

miR-29b is down-regulated in NSCLC tissues
Quantitative RT-PCR results revealed that the expression levels of miR-29b were significantly higher in the H460 and 95C cell lines compared to 16HBE cell line, while the expression levels were lower in the PGCL3, PAA, H520, A549, H1299 and 95D cell lines (Fig. 2a). Twenty pairs of paraffin-embedded NSCLC tissues and normal tissues (Fig. 2b) and ten pairs of fresh NSCLC tissues and normal adjacent tissues (Fig. 2c) were also chosen to detect the expression levels of miR-29b, the results showed that the expression level of miR-29b in twenty cases of paraffin NSCLC tissues was (-1.893 ± 1.367), significantly lower than that in the adjacent lung tissue (-0.605 ± 0.639); P = 0.001, t = -3.817). The expression level of miR-29b in ten cases of fresh non-small cell lung cancer tissues was (-1.996 ± 0.460), significantly lower than that in the adjacent lung tissue (-0.463 ± 0.257; P < 0.001, t = -9.016).

Data present from Table 1 showed the clinicopathologic characteristics of miR-29b expression in NSCLC patients. There was no significant relationship of miR-29b expression with age (P = 0.578), gender (P = 0.862), histology (P = 0.625) and differentiation (P = 0.891); while miR-29b expression was found had significant relationships with lymphatic metastasis (P = 0.004) and clinic stage (P = 0.031). Spearman rank correlation analysis was applied to analyze the expression levels of miR-29b, tumor stage and lymphatic metastasis in NSCLC tissues. The expression of miR-29b was positively correlated with lymphatic metastasis (r = -0.547, P = 0.043). Based on our findings, the expression of miR-29b is down-regulated in NSCLC compared to normal tissues and significantly associated with metastasis.
Fig. 1 Integrated method for screening potential miRNAs and target genes related to NSCLC metastasis. Dendrogram of differentially expressed miRNAs (a) and metastasis-related genes (b) between primary cultured CD133-positive/negative lung adenocarcinoma cells. (c). The overlap of target genes predicted by microRNA.org, TargetScan and Pictar databases.
miR-29b suppresses cell proliferation, migration and invasion in A549 cells

Cell proliferation, migration, and invasion are key steps in tumor metastasis required by tumor cells for metastatic progression in target microenvironments. In A549 cells, after miR-29b lentivirus and negative control (NC) infection, a visible significant difference was seen with fewer miR-29b transfected cells counted than NC transfected cells in migration and invasion assays (Fig. 3a). Consistently, compared with negative controls, the proliferation abilities of A549-miR-29b cells were significantly decreased (Fig. 3b, *P < 0.05, **P < 0.01). Nude mice xenograft model was subsequently applied to evaluate the effect of miR-29b on tumorigenicity (Fig. 3c). Statistical analysis of the mean tumor volume (cm$^3$) demonstrated that miR-29b lentivirus infection inhibited the tumor growth comparing to the control groups (Fig. 3d, **P < 0.01). These findings might indicate that upregulation of miR-29b had a potential to inhibit of metastasis of NSCLC.

miR-29b deficiency alters the metastasis ability of H460 cells

In the present study, we have observed an apparent endogenous expression of miR-29b in H460 cells. Therefore, miR-29b silencing with antisense oligonucleotides...
was administrated in H460 cells. Figure 4a showed that cell migration and invasion ability was promoted in miR-29b inhibitor group comparing to the control groups. miR-29b inhibitor also increased H460 cells proliferation in a time-dependent manner (Fig. 4b, *P < 0.05, **P < 0.01). Compared to H460 cells or H460-LV-NC cells group, tumor growth rates and tumor volumes of H460-LV-miR-29b-inhibitor cells group were significantly increased (Fig. 4c, d, *P < 0.05). Collectively, these observations suggested that miR-29b suppressed growth and metastasis of NSCLC cell in vitro and in vivo.

Effect of miR-29b on cell migration and invasion ability in A549-L and A549-H cells
High-invasion (A549-H) and low-invasion (A549-L) NSCLC cell sublines from A549 cells were created by using the 10 times repeated transwell assay. Herein, the role of miR-29b in cell migration and invasion was evaluated these two kinds of cells. miR-29b was found down-regulated in A549-H cells and up-regulated in A549-L cells. The images showed that miR-29b overexpression inhibited A549-H cells migration and invasion (Fig. 5a). The result of statistical analysis was showed in Fig. 5b (*P < 0.05). As expected, miR-29b inhibitor promoted cell migration and invasion (Fig. 5c) of A549-L cells. The result of statistical analysis was showed in Fig. 5d (*P < 0.05). These data confirmed that miR-29b was a metastasis suppressor in NSCLC cells.

MMP2 as a target gene of miR-29b in NSCLC
To further explore the mechanisms of miR-29b which suppresses lung cancer cell invasion and metastasis, we analyzed probable downstream tumor metastasis-related genes. Computational prediction was used to find out the most likely target genes. The targets of miR-29b were analyzed by the TargetScan, PicTar and MiRanda databases. The analysis revealed that miR-29b bound to the MMP2 3’ UTR with a partially complementary pattern (Fig. 6a). After A549 cells were infected with lentivirus LV-miR-29b, the expression of miR-29b was increased significantly compared with the NC and Blank groups (Fig. 6b, *P < 0.05). As expected, the expression of MMP2 mRNA was down-regulated significantly (Fig. 6c, *P < 0.05). Moreover, western blot results
showed that MMP2 protein expression was decreased significantly (Fig. 6d). Similar results were obtained when miR-29b inhibitor was transfected into the H460 cells, the miR-29b expression level decreased and MMP2 mRNA was up-regulated significantly after 48 h transfection compared with the NC group and Blank groups (Fig. 6b, c, *P < 0.05). MMP2 protein expression was increased after 72 h transfection (Fig. 6d). These findings indicated that miR-29b regulated MMP2 expression negatively. To verify whether MMP2 is a direct target of miR-29b, the 3′UTR of MMP2 cDNA was cloned into the downstream region of the luciferase reporter gene (psiCHECK-2-Wt-MMP2-3′UTR) and co-transfected this vector into 293-T cells with miR-29b mimic (Additional file 5: Figure S1A, B). The luciferase reporter gene study further confirmed that miR-29b bound directly to wild-type MMP2 3′UTR to inhibit the luciferase activity. To confirm the sequence-specific repression of miR-29b, we designed mutated versions of psiCHECK-2-Wt-MMP2-3′UTR carrying 4-bp substitutions in miR-29b target site (psiCHECK-2-Mut-MMP2-3′UTR) (Additional file 5: Figure S1C, D). There was no inhibition effect on the following site-specific mutagenesis of the miR-29b MMP2 3′ UTR binding sites (Fig. 6e, *P < 0.05), indicating that miR-29b directly regulated the target gene MMP2 negatively in NSCLC.

miR-29b affected PTEN expression by binding directly with the PTEN 3′ UTR

TargetScan indicated that miR-29b had two highly conserved PTEN 3′ UTR binding sites (Fig. 7a). Luciferase reporter activity assay was used to determine whether miR-29b regulates PTEN directly via the software-predicted binding sites. Figure 7b depicted the insertion sites of wild-type and mutant PTEN 3′ UTR into the constructed plasmids. The 1500-bp fragment of the 3′UTR region of PTEN mRNA that included the predicted miR-29b recognition site was subcloned and inserted into a luciferase reporter plasmid (Additional file 6: Figure S2A, B). Two miR-29b binding sites in the 3′UTR region of PTEN were mutated to obtain psiCHECK-2-Mut1-3-PTEN-3′UTR plasmid (Additional file 6: Figure S2C–F). Following detection, the luciferase activity in the co-transfected with wild-type PTEN-luc...
reporter and miR-29b mimic group was significantly decreased compared with that in the blank or NC groups (*P < 0.05). The luciferase activity in the co-transfected with mutant-type1 or mutant-type2 PTEN-luc reporter and miR-29b mimic group was also significantly decreased compared with that in the blank or NC groups (*P < 0.05). However, there were no significant changes in the co-transfected with mutant-type3 PTEN-luc reporter and miR-29b mimic group and the blank or NC groups (P > 0.05). This result proved that miR-29b bound directly to both PTEN 3’-UTR binding sites. Therefore, PTEN was a direct target of miR-29b. The influence of miR-29b on PTEN mRNA and protein expression levels were also evaluated in the A549 and H460 cells. There were no obvious changes on PTEN mRNA and protein expression levels in LV-miR-29b infected A549 cells or in miR-29b inhibitor transfected H460 cells compared with the NC or Blank groups (Fig. 7d, e).

**Discussion**

In the present study, we provided evidence that miR-29b expression in high-metastatic CD133-positive A549 lines was down-regulated when compared to miR-29b expression in paired low-metastatic CD133-negtive A549 cell lines, miR-29b was confirmed directly targeted 3’-UTR of PTEN and MMP2 mRNAs and down-regulated MMP2 protein expression to suppress lung cancer metastasis *in vitro* and *in vivo*.

As a MMP superfamily member, MMP2 specifically degrades type IV collagen, a major component of the extracellular matrix and basal lamina, and is a major factor in tumor invasion and angiogenesis [19]. It has been demonstrated that high MMP2 expression is an independent prognostic factor in NSCLC and is closely related to clinical stage, pathological grade, lymphatic metastasis, and prognosis [20]. The regulatory mechanisms of a miRNA could differ among different microenvironments, miR-29b is upregulated in metastatic breast cancer tissues and indolent lymphocytic leukemia, functioning as an oncogene [21, 22]. However, miR-29b is down-regulated in lung carcinoma tissues [23]. In our study, low-level expression of miR-29b in NSCLC tissues was significantly associated with lymphatic metastasis. We performed gain-of-function in A459 cells and loss-of-function in H460 cells of miR-29b. Our data demonstrated that miR-29b inhibited *in vitro* cell proliferation, invasion and migration and *in vivo* suppressed NSCLC.
growth in a nude mice xenograft model. Furthermore, the dual-luciferase reporter assay demonstrated that miR-29b inhibited the expression of luciferase gene containing the 3'-UTR of PTEN and MMP2. Western blotting indicated that miR-29b down-regulated the endogenous protein expression of MMP2. Based on these results, it's concluded that miR-29b was related to metastasis in NSCLC.

PTEN regulates tumor cell growth, cell cycle, apoptosis, and metastasis by regulating multiple signal transduction pathways negatively [24, 25]. All three databases used (TargetScan, PicTar, miRanda) identified the two PTEN 3'-UTR miR-29b binding sites. Both sites were conserved among different species and fully complementary to the miR-29b seed sequence, corresponding to the basic rules for predicting miRNA target genes [26]. Our present results showed that miR-29b bound directly to the two PTEN 3' UTR binding sites and PTEN was a miR-29b target gene. Through miR-29b overexpression or knockdown analysis, the fact was determined that miR-29b variations were not accompanied with the alteration of PTEN expression. As multiple miRNAs could regulate the same target gene [27], we speculate that other miRNAs could also bind directly to the PTEN 3' UTR and regulating PTEN expression. Several research reported that PTEN function as a target gene of miR-21 [28], miR-214 [29], miR-494 [30], miR-26a [31], miR-144 [32] and miR-153 [33]. Of these, miR-21, miR-214, and miR-494 are upregulated in NSCLC. Another reason that might explain our contrasting findings was that
miR-29b directly inhibits CDC42 and p85α to activate p53 expression [34]. P53 activates PTEN transcription, binding directly to the PTEN promoter and activating PTEN expression [35]. PTEN gene was not only indirectly regulated by miR-29b-p53-PTEN positively, but also directly regulated by miR-29b negatively. The inhibition of Sp1 by miR-29b resulted in the upregulation of PTEN in tongue squamous cell carcinoma [36].

**Conclusion**

In summary, our studies demonstrated that down-regulated miR-29b expression was found to be associated with increased MMP2 expression in CD133-positive NSCLC cells through microarrays and bioinformatics analysis. miR-29b played a strong inhibitory role in tumor metastasis. We provided important evidence that miR-29b could suppress NSCLC cells proliferation, migration and invasion by targeting the 3’-UTR of MMP2 and PTEN mRNA to down-regulate MMP2 protein expression. Our findings provided novel evidence for the involvement of miR-29b in NSCLC metastasis, and suggested that miR-29b could be a potential new target for treatment of NSCLC metastasis.

**Consent**

The patient consent of Written informed consent was obtained from the patient for the publication of this report and any accompanying images.
Additional files

Additional file 1: Supplementary Materials and Methods.

Additional file 2: Table S1. Sequences of RNA and DNA Oligonucleotides.

Additional file 3: Table S2. Fifty-one miRNAs differentially expressed in CD133+ A549 cells versus CD133- A549 cells.

Additional file 4: Table S3. Changes in relative expression for tumor metastasis genes between CD133+ and CD133- A549 cells.

Additional file 5: Figure S1. Construction of mutant 3'UTR-MMP2-luc vector.

Additional file 6: Figure S2. Construction of mutant 3'UTR-PTEN-luc vector.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

YS-Z conceived the project and designed the experiments. HY-W and XY-G carried out the majority of the experiments. HY-W and HY-G conducted the bioinformatics analysis. SY-T, SQ-Z and JL helped to collect clinical samples. SH-L, CL-Q, HQ-Z and XB-X helped to culture cells, all authors discussed the results. HY-W and XY-G wrote the manuscript. All authors read and approved the final manuscript.

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