Suppression of Tumor Cell Invasiveness and *In Vivo* Tumor Growth by MicroRNA-874 in Non-Small Cell Lung Cancer

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Abbreviations: NSCLC: non-small cell lung cancer; CSC: cancer stem cell; NL: normal lung; LC: lung cancer; miR-874: microRNA-874

Abstract

MicroRNAs are a novel family of small non-coding RNAs that regulate the expression of several genes involved in normal development as well as human disorders including cancer. Here we show that miR-874 plays a tumor suppressor role in non-small cell lung cancer (NSCLC) *in vitro* and *in vivo*. *In silico* target prediction analysis revealed numerous genes associated with tumor progression including MMP-2 and uPA as the putative target genes of miR-874. Our preliminary *in situ* hybridization experiments demonstrated the diminution of miR-874 expression in lung cancer tissues compared to their normal counter parts. Overexpression of miR-874 in CD133-positive cancer stem cell (CSC) population led to a significant loss in CSC-phenotype and enhanced sphere de-differentiation into epithelial-like cells. Restoration of miR-874 expression drastically reduced cell invading ability in comparison to mock and control miR-treated cells by suppressing the protein levels of MMP-2 and uPA. In *in vivo* experiments, miR-874 treatment decreased orthotopic tumor growth in nude mice compared to mock and control-miR treatments. Further, the immunereactivity of human anti-MMP-2 and anti-uPA was significantly reduced in tumor sections from mice that received miR-874 treatment. In conclusion, our study highlights the possible tumor suppressor role of miR-874 in NSCLC-initiating cells and suggests miR-874 as a potential target in the treatment of NSCLC.

Key words: CSC, invasion, lung cancer, miR-874, MicroRNA, NSCLC.

Introduction

Despite using advanced therapies followed by surgical resection, lung cancer is the major cause of cancer-related mortality in patients worldwide [1] in which non-small cell lung cancer (NSCLC) accounts for ~85%. The primary tumor cells migrate from the site of origin, invade the neighboring tissues and enter the circulation to establish secondary tumors during metastatic dissemination [2]. A large body of reports suggests the role of extracellular signaling molecules present in the regulation of migratory properties of tumor cells. Elevated tumor invasion and migration are, in part, attributed to the abundant expression and availability of cysteine, serine and metalloproteases in the extracellular milieu, which selectively degrade the neighboring extracellular matrix components [3,4]. Recently accumulating evidences show that a small subpopulation of self-renewing stem-like cells called cancer stem cells (CSCs) may be responsible for the initiation, maintenance, progression and metastatic spread of tumors [5]. CSCs are relatively quiescent and possess intrinsic resistance towards conventional chemotherapy and radiotherapy and thus are responsible for tumor recurrence [6]. The poor prognosis in lung cancer patients is primarily attributed to metastatic dissemination and resistance of tumor cells to chemotherapy-the two important traits ascribed to CSCs. The therapeutic strategies aimed at eradicating CSCs may improve patient clinical outcome. However, we still do not have a good understanding of the molecular mechanisms involved in tumor CSC maintenance.

MicroRNAs (miRNA) are a novel family of small, abundant 19-25 nucleotide non-coding RNAs, which regulate translational efficiencies of target genes and play a crucial role in the normal developmental processes, differentiation and in tumorigenesis [7]. Aberrant expression and imperfect pairing of miRNAs with >30% of total protein-coding genes potentially regulate

their expression at transcriptional or post-transcriptional level [8]. MicroRNAs are implied to influence cell proliferation, apoptosis, chemo- and radiation-sensitivity, metastasis, and EMT and could even potentially define the CSC phenotype in cancers [7,9]. With deregulated expression in several cancers including lung cancer, miRNAs are evolving as potential diagnostic and therapeutic markers [7,10-11]. Recently, the tumor suppressive role of miR-874 by inhibition of cell proliferation and invasion was reported in maxillary sinus squamous cell carcinoma [12]. However, the potential role of miR-874 in the regulation of NSCLC tumorigenesis is still unknown. In this study, we demonstrate the effect of miR-874 in NSCLC-initiating cells (NSCLC-ICs) in inhibiting invasion, migration and CSC-phenotype by regulation of MMP-2 and uPA proteins *in vitro* and *in vivo,* suggesting the therapeutic significance of miR-874 in NSCLC treatment.

1. Materials and methods

1.1. Cell culture, reagents and antibodies

A549 and H1299 cell lines were obtained from ATCC (Manassas, VA) and cultured in RPMI 1640 supplemented with FBS (Invitrogen, Carlsbad, CA), 50-units/mL penicillin and 50 μg/mL streptomycin (Life Technologies, Frederick, MD). CSC spheres were obtained from parental A549 and H1299 cells by culturing in knockout DMEM/F12 medium containing N2, B27, bFGF, EGF and LIF as described earlier [13]. Primary spheres were obtained after 5-6 days and were expanded by mechanical dissociation to obtain single cell suspension, which was then plated for secondary sphere formation. Oncospheres up to 5-10 passages were used and designated as A549S and H1299S in the present study. Specific antibodies against CD133, MMP-2, uPA, ALDH1A1, GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), Oct-4 (Cell Signaling

Technology, Danvers, MA), and phycoerythrin-conjugated anti-human CD133 (CD133-PE) (Miltenyi Biotech Inc., Auburn, CA) were used in this study.

2.2. In silico analysis

Putative miR-874 binding sites in 3'-UTRs of various target genes including MMP-2 and uPA were identified using target prediction algorithms, miRanda and TargetScan. Human miR-874 sequences of the RFAM miRNA registry were downloaded from the miRBase website [14].

2.3. CD133 cell sorting

The A549S and H1299S spheres were mechanically disrupted by trituration into single cell suspension and incubated for 60 min at 4 ℃ with anti-CD133-PE following the manufacturer's instructions. The cells were re-suspended in 0.5 mL 1% BSA in PBS, analyzed and sorted by flow cytometry. Acquisition was performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA), and viable cells were analyzed with CellQuest software.

2.4. Plasmid constructs and transient transfection studies

Lentiviral constructs pLV-[hsa-mir-874] and pLV-[hsa-mir-control] plasmids were obtained from Biosettia, Inc. (San Diego, CA). Transient transfection experiments were performed using Fugene HD transfection reagent as per the manufacturer's instructions (Roche, Indianapolis, IN). Cells were collected after 36 hrs of transfection for further analysis.

2.5. RT-PCR and real time-PCR

Total RNA from cell lines was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), and the cDNA was synthesized from 1 μg of total RNA using Transcriptor First Strand cDNA Synthesis kit (Roche, Indianapolis, IN) following the manufacturer's instructions. Semi-quantitative RT-PCR or real-time (qPCR) analyses were performed as described previously [15] using cDNA and

the following set of primers: MMP-2-sense: 5'-GCAGTGCAATACCTGAACACCTTC-3', MMP-2-antisense: 5'-CCATACTTCACACGGACCACTTG-3', uPA-sense: 5'-AGAATTCACCACCATCGAGA-3', uPA-antisense: 5'-ATCAGCTTCACAACAGTCAT-3', GAPDH-sense: 5'-CGGAGTCAACGGATTTGGTCGTAT-3', and GAPDH-antisense: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'. The PCR data obtained from three experimental replicates were analyzed and normalized to the GAPDH control. For miR-874 expression analysis, we obtained the miRCURY LNA™ Universal RT microRNA PCR system from Exiqon (Woburn, MA), and real time PCR was performed according to the manufacturer's instructions. Data were analyzed using iCyclerIQ version 3.1 software (Bio-Rad). The Ct values were converted into fold change of expression using 2-ΔΔCt method (where ΔΔCT = ΔCT of treatment –ΔCT of control), and data were normalized to internal U6 controls.

2.6. Immunoblotting and zymography

Immunoblotting was performed using whole cell lysates as described previously [16]. At 36 hour post-transfection, serum-free medium treated NSCLC-ICs and incubated for another 16 hrs. Tumor conditioned media were collected and subjected to gelatin and fibrin zymographic analysis to determine MMP-2 and uPA activities, respectively [17,18]

2.7. Sphere de-differentiation and matrigel invasion assay

The mock and miR-874-transfected NSCLC-ICs were cultured to form secondary spheres as described earlier [19]. After 5 days, sphere formation was observed and visualized using phase contrast microscopy. The NSCLC-ICs were treated as described above, and *in vitro* Transwell matrigel invasion assay was performed as explained previously [18].

2.8. In situ hybridization (ISH)

The miRCURY LNA™ microRNA ISH Optimization Kit (FFPE) and full-length miR-874 hybridization probe were purchased from Exiqon (Woburn, MA). ISH was performed in orthotopic tumor sections and human lung cancer tissue array (Panomics, Richmond, CA) comprised of NSCLC lung cancer tissues (LC, n = 36) and normal lung tissues (NL, n = 4) in duplicate cores as per the manufacturer's instructions [20]. Briefly, the de-paraffinized tissue sections were treated with proteinase-K, subjected to pre-hybridization and followed by hybridization with DIG-labeled LNA miR-874 probe. Sections were washed with SSC buffers and blocked by DIG blocking reagent, incubated with alkaline phosphatase-conjugated anti-DIG, and developed using alkaline phosphatase substrate followed by nuclear fast red counterstaining. The slides were mounted and images were captured.

2.10. In vivo orthotopic tumor experiments and Immunohistochemistry

A549S cells (1×10^5) were surgically injected to establish *in vivo* orthotopic tumor growth in athymic nude mice [21]. The mice with tumors were divided into three groups to receive tail vein injections of mock, control-miR and miR-874 (3-6 mg/kg body weight) in three doses (7^{th}, 9^{th} and 11^{th} days). On day 40, mice were euthanized and excised tumors were fixed in paraformaldehyde. De-paraffinized lung tumor sections were stained with hematoxylin and eosin, and tumor volume was calculated as described previously [18]. Immunohistochemical analysis was performed by incubation with specific antibodies against MMP-2 and uPA followed by HRP-conjugated secondary antibodies. Sections were subjected to DAB (3,3'-diaminobenzidine) staining, nuclei were counterstained with hematoxylin, mounted and photographed.

2.11. Statistical analysis

Densitometric analysis was performed using ImageJ software (NIH). Values are expressed as mean of triplicate experiments ± SD and differences were considered significant at a $p < 0.05$.

3. Results

3.1. Loss of miR-874 expression in NSCLC

Here, we generated the precursor miR-874 stem-loop structure using mFold software (http://mfold.rna.albany.edu). Figure 1A shows the predicted RNA stem-loop hairpin structure of pre-miR-874 registered in miRNA database (http://www.mirbase.org/). The two mature miR-874-5' and miR-874-3' forms formed a stem in a predicted stem-loop hairpin structure, which is a signature of the miRNA precursor. Based on recent evidence suggesting the tumor suppressor role of miR-874 [12], we attempted to evaluate basal miR-874 expression levels in NSCLC tissue specimens through ISH in lung cancer tissue. The lung cancer tissues (LC; n = 36) showed very little or no detectable miR-874 levels in comparison to normal lung (NL; n = 4) control tissues, which displayed substantial levels of endogenous miR-874 (Figs.1B-C). Total scores of cellular miR-874 positivity showed significant reduction in LC tissues compared to NL and suggested the inverse correlation between miR-874 and NSCLC progression (Fig.1C). Further, computational target prediction analysis indicated a significant number of essential genes involved in tumor angiogenesis, invasion and migration including MMP-2, uPA, MAP3K1, VEGF-A, Stat3 etc., as putative target genes of miR-874 (Table-1). The putative target sites for miR-874 target sites in the 3'-UTRs of MMP-2 and uPA are shown in Figure1D.

3.2. Isolation of A549 and H1299 CSC population

Increasing evidence demonstrate the occurrence of a small subpopulation of cells called CSCs or cancer-initiating cells with self-renewal ability, which are responsible for tumor metastasis and

recurrence [22]. With the above data providing novel insights into the possible role of miR-874 in the regulation of essential molecular intermediates participating in tumor metastasis, we next examined the effect of miR-874 overexpression on CSC phenotype, invasion and migration in NSCLC-ICs. To achieve this, we isolated CD133-positive A549 and H1299 cells with CSC characteristics and designated as A549S and H1299S. Phase contrast microscopy revealed the characteristic sphere forming ability of the CD133-positive cells whereas CD133-negative cells did not form spheres (Fig.2A). Enhanced CSC characteristics of A549S and H1299S cells were further confirmed by immunoblot analysis for NSCLC stem cell markers CD133, Oct-4 and ALDH1A1 expression (Fig.2B).

3.3. Overexpression of miR-874 led to sphere de-differentiation into epithelial-like cells and inhibited invasion in NSCLC-ICs

NSCLC-ICs were treated with mock, control-miR and miR-874 for 36 hrs, and stem-loop quantitative RT-PCR was performed to evaluate miR-874 levels. The miR-874-treated cells showed significantly high expression of miR-874 when compared to the corresponding controls (Fig.3A). The sphere de-differentiation assay indicated continued growth of mock and control-miR cells in the form of spheres after treatment (Fig.3B). On the other hand, miR-874-treatment resulted in sphere de-differentiation and increased epithelial-like morphology and adhering ability compared to corresponding controls. These results suggest that the overexpression of miR-874 increased epithelial characteristics and decreased self-renewing capacity in NSCLC-ICs. Furthermore, Transwell invasion assays revealed the impeded invasive potential of miR-874-treated cells (> 70%) suggesting that miR-874 decreases cell motility and invasion through ECM in these cells (Fig.3C).

Various cancers including lung cancer show increased expression of MMP-2 and uPA [23-27]. Since both MMP-2 and uPA were reported to be involved in tumor cell invasion, migration and angiogenesis [23,24,26-28] and also possess putative target sites for miR-874 in their 3'UTR, we analyzed the expression levels of MMP-2 and uPA in miR-874-transfected NSCLC-ICs. We observed a significant decrease in transcriptional levels of MMP-2 and uPA by ~80% in miR-874 overexpressing cells (Fig.3D). In corroboration, the protein levels of MMP-2 and uPA were decreased by >70% in miR-874-treated cells (Fig.3D). Additionally, we also observed a significant decrease in MMP-2 gelatinolytic activity and uPA fibrinogen-degrading activity in miR-874-overexpressed NSCLC-ICs as compared to their mock and control-miR control counterparts.

3.4. miR-874 inhibited in vivo orthotopic tumor growth

We performed *in vivo* experiments to evaluate the effect of miR-874 on orthotopic tumor growth by intrathoracic implantation of A549S cells in mice (Fig.4A). Mock or control-miR-treated animals showed weight loss and behavioral changes, such as inability to feed properly and lameness due to tumor burden. On the other hand, miR-874-treated animals remained symptom-free. Representative micrographs of lung tumors in mock, control-miR and miR-874 treatments were taken and indicate significant reduction in the growth of miR-874-treated tumors (Fig.4B). The Hematoxylin and Eosin staining sections of A549S orthotopic tumors indicate tumor growth pattern in different treatments (Fig.4B). The relative tumor size was significantly reduced in miR-874-treated animals when compared to mock and control-miR-treatments (Figs. 4B and C). Further, ISH analysis confirmed the expression levels of miR-874 in orthotopic tumor sections (Fig.4D). A prominent inhibition in MMP-2 and uPA expression levels was evident in miR-874-treated cells (Fig.4E). On the other hand, the mock and control-miR-treated tumors displayed

high MMP-2 and uPA levels. These results suggest that restoration of miR-874 alleviated the expression of MMP-2 and uPA, and thus inhibited tumor growth.

4. Discussion

Even though surgical resection is the most optimal treatment, NSCLC, a sub-type of lung cancer, has about 73% (1^{st} stage) and 15% five-year survival rates in patients. These rates highlight the importance of attaining a better understanding of the molecular mechanisms involved in disease progression. CSCs in bulk tumors are potentially resistant to standard therapies and are primarily responsible for tumor initiation, progression, recurrence and metastasis [6,14]. CSCs share characteristic features of self-renewal and ability to differentiate into heterogeneous lineage in embryonic and somatic stem cells and possess distinct cell surface markers including CD44, CD133, CD24 and ESA when cultured in *in vitro* conditions [22]. In the present study, we effectively isolated and established the CSC cultures, A549S and H1299S. Of note, sphere-derived NSCLC-ICs displayed significant expression levels of MMP-2 and uPA.

Numerous studies suggest the possible oncogenic or tumor suppressive roles of microRNAs through the regulation of several genes that are necessary for invasion and migration in cancer [29,30]. The differential expression and critical tumor suppressive roles of several miRNAs including miR-32, miR-128b, miR-103, miR-107, miR-200c, miR-221, and miR-222, have been reported in lung cancer [31]. Similarly, multivariate analysis in NSCLCs identified that reduced miR-155 and let-7 significantly correlated with a shorter patient survival rate [32,33]. The chromosomal fragile sites are heritable specific loci prone to breaks and are denser in microRNAs and protein-coding genes in several human pathologies, particularly cancer [34]. miR-874 is located on chromosome 5q31.2, a well-known frequent fragile site in the human genome often deleted in cancers and genetic disorders and specifically correlated with

chromosomal rearrangements in cancer [35,36]. We confirmed miR-874 expression levels in NSCLC tumor samples and normal tissues by ISH analysis, which displayed remarkable loss in the NSCLC tissues, thereby suggesting the potential tumor suppressive role of miR-874. To our knowledge, this is the first report investigating aberrant miR-874 expression in NSCLC tissue samples.

Our *in silico* analysis showed highly conserved sequence complementary to the seed sequence of miR-874 was identified in a group of target genes including extracellular proteases MMP-2 and uPA. MMP-2 is a cellular membrane-bound and extracellular protease found to be aberrantly expressed in lung cancer and is associated with elevated tumor invasion [24,37]. Several studies suggest that MMP-2 and uPA are critical extracellular signaling molecules that transmit the outward-to-inward oncogenic signals in an integrin-dependent manner and regulate cell survival, invasion and angiogenesis in tumors [26-28]. In the present study, ectopic expression of miR-874 resulted in suppression of MMP-2 and uPA activities in sphere-derived NSCLC-ICs and subsequently decreased invasion in these cells. Overexpression of miR-874 also resulted in loss of CSC phenotype and led to acquiring epithelial-like sphere de-differentiation. Sphere-derived NSCLC-ICs displayed significant expression levels of MMP-2 and uPA in correlation with *in vivo* tumorigenic potential. Finally, miR-874 significantly decreased tumor growth. Consistent with the *in vitro* results, the immunoreactivity of anti-MMP-2 antibody and anti-uPA were decreased in tumor tissue sections from mice that received miR-874 treatment.

In summary, our study elucidates the role of miR-874 downregulation in NSCLC progression and suggested that the frequent loss of miR-874 leads to increased tumor malignancy. Our *in vivo* studies of decreased orthotopic tumor growth in miR-874-treated mice

confirmed the *in vitro* findings and suggest the potential therapeutic application of miR-874 in NSCLC patients.

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Fig.1. Diminished miR-874 expression in NSCLC (A) The predicted stem-loop structures of pre-miR-874. The mature miR-874 is also shown. (B) *In situ* hybridization (ISH) was performed on a lung cancer tissue array using DIG-labeled locked nucleic acid (LNA)-based probes for miR-874. Control probe used: U6. Representative micrographs for miR-874 expression in normal lung (NL) and NSCLC tissues (LC) are shown (10X, top). Inset portions were enlarged (40X, bottom). (C) Total scores of miRNA-874 cellular-positivity were determined from randomly chosen microscopic fields. The mean (indicated by solid line) values normalized to U6 scores (*$p<0.01$) (D) Putative target sites for miR-874 in 3'-UTR of MMP-2 and uPA mRNA.

Fig.2. Isolation and confirmation of CSCs. (A) CD133-positive and CD133-negative cells were cultured as described in Materials and Methods. Representative micrographs of sphere formation are shown. (B) Total cell lysates were subjected to immunoblotting to determine NSCLC stem cell markers CD133, Oct-4 and ALDH1A1 expression levels. GAPDH was used as a loading control.

Fig.3. Overexpression of miR-874 led to sphere de-differentiation and inhibited invasion in NSCLC-ICs. The NSCLC-ICs were transfected with control-miR and miR-874 as described in Materials and Methods. (A) At 36hrs post-transfection, qPCR was performed for analyzing miR-874 levels. Data was normalized to internal U6 controls. *Column*: mean; *bar:* ± SD (n=3; $p<0.01$ Vs. Control-miR). (B) After 5 days of transfection with miR-874, sphere de-differentiation ability of CD133-positive cells into epithelial-like cells were photographed. (C) At 36hrs post-transfection, cells were disrupted into single-cell suspension by trituration and were added onto Matrigel-coated Transwell inserts. After 16hrs, invaded cells were stained. Representative micrographs and relative percentage of invaded cells were shown from three replicates. (D) At 36-hour post-transfection, the mRNA and protein expression levels of MMP-2 and uPA were

determined by RT-PCR and immunoblotting, respectively. GAPDH served as a loading control. Gelatin (MMP-2) and fibrin (uPAR) zymographic analyses show activities of MMP-2 and uPA in conditioned medium from miR-874-overexpressed cells.

Fig.4. miR-874 suppressed *in vivo* A549S orthotopic tumor growth in nude mice. (A) Schematic representation of A549S tumor cell intrathoracic implantation and miR-874 treatment. (B) Representative lung micrographs from mock, control-miR and miR-874-treated mice are shown. Also shown H&E stained lung tissue sections (2.5× and 60×). (C) The relative lung tumor size is represented as mean±SD, (n=6). (D) *In situ* hybridization analysis was performed in mock, control-miR and miR-874-treated tumor sections to determine miR-874 expression. (E) Immunohistochemical analysis in lung tumor sections was performed as explained in Materials and Methods. Representative micrographs for MMP-2 and uPA expression are presented. Inset: Isotype control.

Table1. miR-874 putative target genes those involves in tumor progression.