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Comprehensive identification of microRNA arm selection preference in lung cancer: miR-324-5p and -3p serve oncogenic functions in lung cancer

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Abstract. MicroRNA (miRNA/miR) dysfunction is a hallmark of lung cancer, and results in the dysregulation of tumor suppressors and oncogenes during lung cancer progression. Selection of the 5p and 3p arms of miRNA is a mechanism that improves the modulation of miRNA biological functions and complicates the regulatory network in human types of cancer. However, the involvement of arm selection preference of miRNA in lung cancer remains unclear. In the present study, changes in miRNA arm selection preference were comprehensively identified in lung cancer and corresponding adjacent normal tissues by analyzing The Cancer Genome Atlas. Arm selection was revealed to be consistent in the majority of miRNAs in lung cancer. Only a few miRNAs had significantly altered arm selection preference in lung cancer. Among these, the biological functions of the individual arms of miR-324 were investigated further. The data revealed that miR-324-5p and -3p were significantly overexpressed in lung cancer cells. Ectopic expression of miR-324-5p significantly promoted cell proliferation and invasion in lung cancer cells, while miR-324-3p overexpression significantly increased cell proliferation but did not alter the invasion of lung cancer cells. In conclusion, the arm selection preference of miRNA may be an additional mechanism through which biological functions are modulated. The results of the present study provide a novel insight into the underlying mechanisms of lung cancer and may direct research into future therapies.

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Introduction

Lung cancer is the leading cause of cancer-associated mortality globally (1). Non-small-cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for ~85% of all lung cancers, and >40% of NSCLC cases are metastatic (stage IV) at the point of diagnosis (2,3). Lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) are the major pathological subtypes of NSCLC. NSCLC treatment has improved in the past decade, with more effective screening, diagnosis, and systemic treatments contributing to increased survival rates (3). Lung cancer cells frequently participate in cancer-associated pathways, including growth signaling, programmed cell death, angiogenesis, cell invasion, and metastasis (4). Transformation from a normal cell to a malignant lung cancer cell phenotype is considered to occur in a multistep manner through a series of genetic and epigenetic alterations; ultimately evolving into invasive cancer by clonal expansion (5). Dysfunctional noncoding RNAs, including microRNAs (miRNAs/miRs), have been reported to be critical in carcinogenesis (6). MiRNAs are small, non-protein-coding RNAs, 19-24 nucleotides in length, which negatively modulate gene expression by suppressing

mRNA translation or accelerating mRNA degradation (7-9). A single miRNA may regulate >100 target genes by targeting their 3'-untranslated regions. Therefore, miRNAs typically only fine-tune mRNA expression, suggesting that co-expressed miRNAs may function simultaneously to co-modulate a signaling pathway to achieve a biological function (10). Numerous dysregulated miRNAs have been reported in lung cancer cells, including let-7 and miR-17, -20a, -29, -107, -126, -138, and -185 (11-17). Dysfunctional expression of these miRNAs serves a critical function in the co-modulation of cell cycle progression, angiogenesis, apoptosis, adhesion and motility of lung cancer cells. In addition to miRNA-mRNA interaction networks, miR-#-5p and -3p arm selection preference is a complex mechanism that regulates the biological functions of miRNAs. Although the 5p and 3p arms of miRNA are generated from an identical pre-miRNA structure during the maturation process, the expression of certain miRNAs is altered in different tissues, developmental stages and species, as well as during cancer progression (18-27). However, the underlying mechanisms and functions of miRNA arm selection preference in lung cancer cells remain unclear. In the present study, multiple miRNA candidates with significantly altered arm selection preference in lung cancer were identified. Among these, miR-324 was selected for further examination in the present study. Our previous study revealed that miR-324-5p and -3p serve tumor suppressive functions in breast cancer and colon cancer (28). However, the function of miR-324-5p and -3p remains unclear in lung cancer. The present study is the first to report that miR-324-5p and -3p serve oncogenic functions in lung cancer cells. The results suggested that miR-324 may be a novel potential therapeutic target. These results may provide novel insights into lung cancer therapy and direct future research.

Materials and methods

Identification of miRNA arm selection preference using The Cancer Genome Atlas (TCGA). A total of 891 small RNA expression profiles of LUAD (46 normal and 458 tumor tissues) and LUSC (45 normal and 342 tumor tissues) were downloaded from TCGA (https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm). A total of 253 miRNAs were selected for further examination of their arm selection preference, with the simultaneous expression of the 5p and 3p arms (expression of miR-#-5p or miR-#-3p >1 transcripts per million) in LUAD and LUSC. The 5p:3p arm expression ratio was assessed in LUAD, LUSC, and adjacent normal tissues. A fold change of >1.5 or <0.75 and $P<0.05$ was considered to indicate a significant difference in the arm selection preference.

Cell lines. A human lung cancer cell line, A549, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (Biological Industries, Cromwell, CT, USA), supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) and penicillin-streptomycin (penicillin, 100 U/ml and streptomycin, 100 μg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in a humidified atmosphere containing 5% CO_2 at 37˚C.

Ectopic expression of miR-324-5p and -3p following transfection with mimics. The A549 cells were seeded in a 25T flask at a density of $1x10^6$ cells/ml and were transfected with 10 nM miRNA-324-5p mimics; sense, 5'-CGCAUCCCCUAGGGCAUUGGUGU-3' and antisense, 5'-ACCAAUGCCCUAGGGGAUGCGUU-3', miR-324-3p mimics; sense, 5'-ACUGCCCCAGGUGCUGCUGG-3' and antisense, 5'-AGCAGCACCUGGGGCAGUUU-3' or a scramble control; sense, 5'-GCGACGAUCUGCCUAAGAUdTdT-3' and antisense, 5'-AUCUUAGGCAGAUCGUCGCdTdT-3' (GenDiscovery Biotechnology, Inc., New Taipei, Taiwan), using Lipofectamine RNAiMAX reagent, according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). After 24 h of transfection, total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. Briefly, tissue samples were homogenized in 1 ml TRIzol reagent and mixed with 0.2 ml chloroform for protein extraction, and RNA was precipitated by adding 0.5 ml isopropanol. The concentration, purity, and total RNA content were determined using a NanoDrop 1000 spectrophotometer (Nanodrop Technologies; Thermo Fisher Scientific, Inc.). The transfection efficiency was examined through stem-loop reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.

Stem-loop RT-qPCR. Total RNA (1 μg) was reverse-transcribed in a stem-loop RT reaction using RT primers and SuperScript III reverse transcriptase, according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.). The RT reaction, included 1 μg RNA, 1X RT buffer (200 mM Tris-HCl, 500 mM KCl; pH 8.4), 2.5 mM dNTP (Invitrogen; Thermo Fisher Scientific, Inc.) and 0.5 mM stem-loop miR-324-5p or -3p-RT primer (Gemomics BioSci and Tech, New Taipei, Taiwan). The reaction was performed under the following incubation conditions: 30 min at 16˚C, followed by 50 cycles of 20˚C for 30 sec, 42˚C for 30 sec and 50˚C for 1 sec. The enzyme was subsequently inactivated by incubation at 85˚C for 5 min. qPCR was performed using an miRNA-specific forward primer and universal reverse primer, and the reaction mixture was incubated at 94˚C for 10 min, followed by 40 cycles of 94˚C for 15 sec and 60˚C for 32 sec. Gene expression was detected using a SYBR Green I assay (Applied Biosystems; Thermo Fisher Scientific, Inc.), and miRNA expression was normalized to that of U6. The primer sequences used to examine miRNAs were as follows: miR-324-5p-RT primer: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACACCAAT-3'; miR-324-5p gene-specific forward (GSF), 5'-CGGCGGCGCATCCCCTAGGGCAT-3'; miR-324-3p-RT, 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCAGCAGC-3'; miR-324-3p-GSF, 5'-CGGCGGACTGCCCCAGGTGC-3'; universal reverse, 5'-CTGGTGTCGTGGAGTCGGCAATTC-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'.

Cell proliferation and colony formation assay. For the clonogenic assay, a gradient number of A549 cells, 1,000, 2,000 and 4,000, were seeded in 6-well plates and transfected with

10 nM miR-324-5p mimics, miR-324-3p mimics or a scramble control as aforementioned. The cells were incubated in a CO_2 incubator at 37˚C for 2 weeks until colony formation. The cells were then fixed with 4% formaldehyde (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature and stained with crystal violet solution (0.05% crystal violet, 1% formaldehyde and 1% methanol; all from Sigma-Aldrich; Merck KGaA) for 20 min at room temperature. Then, the images of a representative colony formation were examined using a light microscope (magnification, x100) (CKX41; Olympus Corporation, Tokyo, Japan). Finally, 1 ml of 10% acetic acid (Sigma-Aldrich; Merck KGaA) was added to each well to dissolve the crystal violet. The absorbance of individual wells was determined at 595 nm by using Multiskan FC (Thermo Fisher Scientific, Inc., Waltham, MA, USA). For the cell proliferation assay, $1x10^3$ A549 cells transfected with 10 nM miR-324-5p mimics, miR-324-3p mimics, or a scramble control were seeded in a 96-well plate. Proliferation was determined at 0, 1, 2, 3, and 4 days using the CellTiter-Glo One Solution assay, according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA). All experiments were independently repeated 3 times.

Cell invasion assays. A549 cell invasion was analyzed using Transwell assays. Briefly, $4.5x10^5$ cells were suspended in a serum-deprived DMEM (Biological Industries), supplemented with penicillin-streptomycin (penicillin, 100 U/ml and streptomycin, 100 μg/ml; Sigma-Aldrich; Merck KGaA) and seeded on the upper chamber of the Transwell plates (Falcon; Corning Inc., Corning, NY, USA) with a Matrigel coating, and DMEM (Biological Industries), supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) and penicillin-streptomycin (penicillin, 100 U/ml and streptomycin, 100 μg/ml; Sigma-Aldrich; Merck KGaA) was added to the lower chambers for the invasion assay. The chambers were incubated in a CO_2 incubator at 37˚C for 12 or 24 h. The remaining cells in the upper chamber were then removed using cotton swabs, and the cells under the surface of the Transwell plates were fixed in 10% formaldehyde solution for 10 min at room temperature. The cells were stained with crystal violet solution (0.05% crystal violet, 1% Formaldehyde and 1% methanol) (Sigma-Aldrich; Merck KGaA) for 20 min at room temperature, and the number of lung cancer cells in 3 fields of view was counted through phase-contrast microscopy. All experiments were repeated 3 times.

Identification of putative target genes of miR-324 with bioinformatics. A total of 82 putative target genes of miR-324-5p were identified using the TargetScan tool (release no. 7.0) (29). The expression levels of these putative target genes in LUAD and LUSC were obtained from TCGA. The correlations between putative target gene and miR-324-5p expression in LUAD or LUSC were examined using Spearman's correlation analysis.

Statistical analysis. All statistical analyses were carried out using the SPSS 22.0 statistical software package (IBM Corp., Armonk, NY, USA). Changes in the arm selection preference of miRNAs in lung cancer cells from TCGA were analyzed using unpaired Student's t-tests. Correlations between the miR-324-5p:-3p ratio in LUAD, LUSC and normal tissues were determined using Pearson's correlation coefficient analysis, with R^2 as indicated. Spearman's correlation analysis was applied to examine the correlations between miR-324-5p and its target gene expression in lung cancer. Data concerning miR-324-5p and -3p expression in paired LUAD or LUSC tissues were analyzed using paired Student's t-tests. Cell proliferation and invasion were assessed in triplicate, and histograms represent the mean ± standard deviation. The data of cell growth and invasion were analyzed using one-way ANOVA. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of changes in candidate miRNA arm selection preference in lung cancer using TCGA. Our group has previously demonstrated that the arm selection preference of certain miRNAs is altered significantly in breast cancer tissues compared with adjacent normal tissues (27). However, the arm selection preference of miRNAs in lung cancer remains to be clarified. To identify candidate miRNAs with an altered arm preference in lung cancer, small RNA profiles of 458 LUAD and 46 adjacent normal tissues, and 342 LUSC and 45 adjacent normal tissues, were downloaded from TCGA. As presented in Fig. 1A, 932 pre-miRNAs were detected in lung cancer (transcripts/million >1). Among these, 253 pre-miRNAs simultaneously produced miR-#-5p and miR-#-3p. The 5p:3p ratio was further analyzed in identical pre-miRNAs, and the 5p:3p ratio of the majority of miRNAs was revealed to be consistent between lung cancer and adjacent normal tissues (LUAD: $R^2=0.9304$ and LUSC: $R^2=0.9188$; Fig. 1B). Only a few miRNAs had significantly altered arm preference in LUAD or LUSC compared with adjacent normal tissues (fold change >1.5 or <0.75; $P<0.05$). In LUAD, the 5p:3p ratio of 73 miRNAs was significantly increased, and the 5p:3p ratio of 56 miRNAs was significantly decreased. In LUSC, the 5p:3p ratio of 71 miRNAs was significantly increased, and the 5p:3p ratio of 46 miRNAs was significantly decreased. Alterations in the arm selection preference of common miRNA candidates were further identified using a Venn diagram tool (version 2.1) (30). The results revealed that the arm selection preference of 36 miRNAs was upregulated and that of 19 miRNAs was downregulated in lung cancer (Fig. 1C; Table I).

miR-324-5p and -3p serve distinctive functions in lung cancer. Our previous study revealed that miR-324-5p and miR-324-3p play distinct biological functions in breast cancer (28). However, the role of miR-324-5p and miR-324-3p was still unknown in lung cancer. Among the miRNA candidates with a switched arm preference, miR-324 was selected for further examination in the present study. According to the miRbase database, the pre-mir-324 gene is located in chr17:7223297-7223379, and an identical pre-mir-324 simultaneously produces miR-324-5p and -3p in humans (31-35). MiR-324-5p and -3p were significantly overexpressed in LUAD and LUSC compared with adjacent normal tissues (Fig. 2A and B). The expression of miR-324-5p was increased compared with miR-324-3p in lung cancer (Fig. 2C). As presented in Fig. 2C, the 5p:3p ratio in miR-324 was significantly increased (~2.2-fold) in LUAD compared

Table I. Candidate miRNAs indicate changes in arm selection preferences in lung cancer tissues compared with normal tissues.

A, Upregulated miRNAs

	5p/3p ratio fold change (tumor/normal)	
miRNAs	Lung squamous cell carcinoma (LUSC)	Lung adenocarcinoma (LUAD)
hsa-mir-9-1	9.2	10.9
hsa-mir-9-2	7.0	14.6
hsa-mir-17	2.7	1.9
hsa-mir-27b	1.8	1.6
hsa-mir-29a	2.5	4.5
hsa-mir-30c-1	2.5	4.7
hsa-mir-30c-2	6.3	8.7
hsa-mir-30e	1.9	2.1
hsa-mir-33a	3.0	9.0
hsa-mir-33b	2.0	14.4
hsa-mir-96	19.8	69.5
hsa-mir-101-1	1.6	2.2
hsa-mir-125b-2	2.0	4.4
hsa-mir-127	2.2	4.5
hsa-mir-130a	1.9	1.9
hsa-mir-136	3.3	2.2
hsa-mir-140	2.1	1.9
hsa-mir-143	3.7	3.2
hsa-mir-149	3.2	3.5
hsa-mir-182	11.2	38.8
hsa-mir-205	8.7	11.3
hsa-mir-218-1	1.8	2.1
hsa-mir-224	4.3	2.4
hsa-mir-324	1.5	2.2
hsa-mir-337	2.0	4.9
hsa-mir-362	2.0	2.9
hsa-mir-425	2.0	1.8
hsa-mir-502	2.1	1.6
hsa-mir-556	3.0	5.4
hsa-mir-616	1.7	5.2
hsa-mir-671	4.3	3.4
hsa-mir-877	5.0	3.0
hsa-mir-2277	1.9	11.1
hsa-mir-3065	2.2	1.7
hsa-mir-3607	1.8	23.6
hsa-mir-3679	6.7	8.6

B, Downregulated miRNAs

	5p/3p ratio fold change (tumor/normal)	
miRNAs	Lung squamous cell carcinoma (LUSC)	Lung squamous cell carcinoma (LUSC)
hsa-let-7a-3	0.6	0.6

Table I. Continued.

B, Downregulated miRNAs

	5p/3p ratio fold change (tumor/normal)	
miRNAs	Lung squamous cell carcinoma (LUSC)	Lung squamous cell carcinoma (LUSC)
hsa-let-7g	0.5	0.1
hsa-mir-20a	0.5	0.7
hsa-mir-26a	0.5	0.7
hsa-mir-27a	0.3	0.4
hsa-mir-29b	0.5	0.1
hsa-mir-138	0.3	0.5
hsa-mir-142	0.6	0.3
hsa-mir-335	0.7	0.5
hsa-mir-374b	0.7	0.6
hsa-mir-423	0.6	0.3
hsa-mir-455	0.2	0.4
hsa-mir-497	0.3	0.7
hsa-mir-509	0.6	0.3
hsa-mir-532	0.5	0.5
hsa-mir-589	0.4	0.6
hsa-mir-629	0.3	0.4
hsa-mir-744	0.3	0.2
hsa-mir-769	0.6	0.4

miR, microRNA.

with adjacent normal tissues. Furthermore, the 5p:3p ratio in miR-324 was significantly increased (~1.5-fold) in LUSC compared with adjacent normal tissues. Therefore, the 5p arm selection of miR-324 was more preferred in tumors compared with adjacent normal tissues. To investigate the biological functions of the individual arms of miR-324, miR-324-5p and miR-324-3p mimics were transfected into the A549 cells. Following transfection, the expression of miR-324-5p and -3p significantly increased in the transfected cells compared with the scramble controls (data not shown). Subsequently, cell proliferation and invasion were examined. As presented in Fig. 3A and B, ectopic miR-324-5p and -3p increased the proliferation of A549 cells. However, only miR-324-5p significantly increased cell invasion in lung cancer cells (Fig. 3C and D). These results revealed that miR-324-5p and -3p were generated from an identical pre-mir-324; however, the arm selection preference of miR-324 differed significantly between lung cancer and normal tissues. Different arms of miR-324 may serve distinct biological functions in the modulation of lung cancer cell invasion.

Identification of putative target genes of miR-324-5p in lung cancer. The data revealed that miR-324-5p significantly promoted lung cancer cell invasion. Therefore, putative target genes of miR-324-5p were identified using a bioinformatics approach. A total of 83 putative target genes of miR-324-5p were identified using TargetScan prediction tools. The

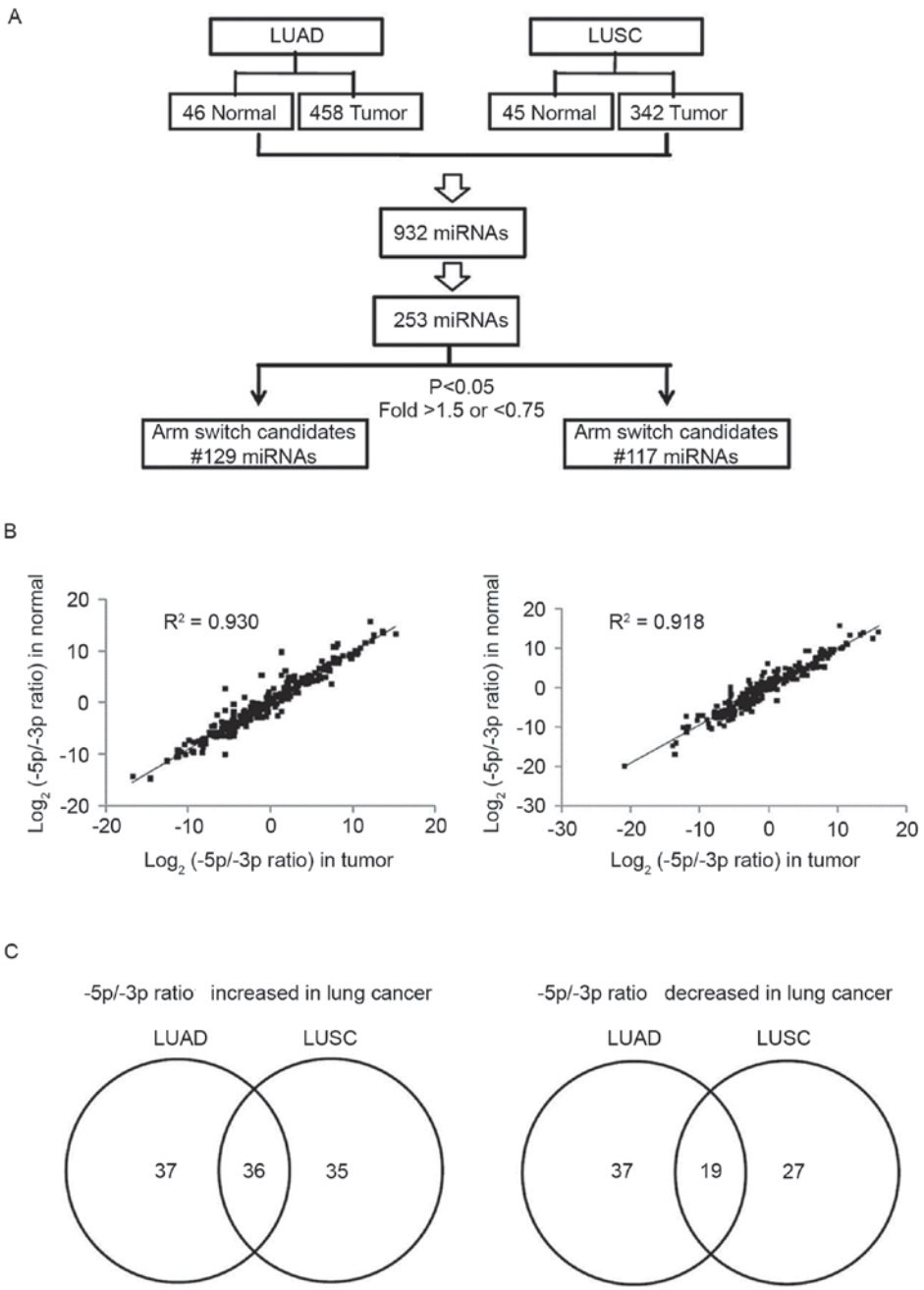


Figure 1. Analysis of miRNA arm selection preference in LUSC and LUAD. (A) Workflow for identifying the changes in the arm selection preference of miRNA candidates in lung cancer. The small RNA expression profiles of LUAD (46 normal and 458 tumor tissues) and LUSC (45 normal and 342 tumor tissues) were downloaded from The Cancer Gene Atlas. The candidates with a significantly (P<0.05) increased (fold change >1.5) or decreased (fold change <0.75) miR-#-5p:-3p ratio in lung cancer tissues compared with adjacent normal tissues were considered as the change candidates. (B) A correlation was observed in the miR-#-5p:-3p ratio of 253 miRNAs between LUAD (left panel) or LUSC (right panel) and normal tissues. (C) Changes in the arm selection preference of common miRNAs from the two data sets (LUAD and LUSC) were identified using a Venn tool. LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma; miRNA/miR, microRNA.

expression levels of the target genes should be negatively correlated with miR-324-5p expression in lung cancer. Among these identified target genes, 11 gene expression levels were negatively correlated with miR-324-5p expression in LUAD and LUSC (Fig. 4A). As the expression levels of miR-324-5p were increased in lung cancer, those of the target genes should be decreased in lung cancer. The expression levels of the predicted candidates were further analyzed using TCGA, which revealed that glutathione peroxidase 3 (*GPX3*), regulator of calcineurin 1 (*RCAN1*) and mannosyl (β-1,4-) glycoprotein β-1,4-*N*-acetylglucosaminyltransferase (*MGAT3*) expression levels were significantly decreased (fold change <0.5) in LUAD and LUSC compared with adjacent normal tissues (Fig. 4B and C). Previous studies have reported that *GPX3*, *RCAN1* and *MGAT3* expression is significantly downregulated and serves a tumor-suppressive function in human cancer, including prostate cancer, lung cancer, ovarian cancer and lymphoma (36-39). According to the aforementioned data, miR-324-5p may promote lung cancer cell proliferation and invasion by suppressing *GPX3*, *RCAN1* and *MGAT3* expression

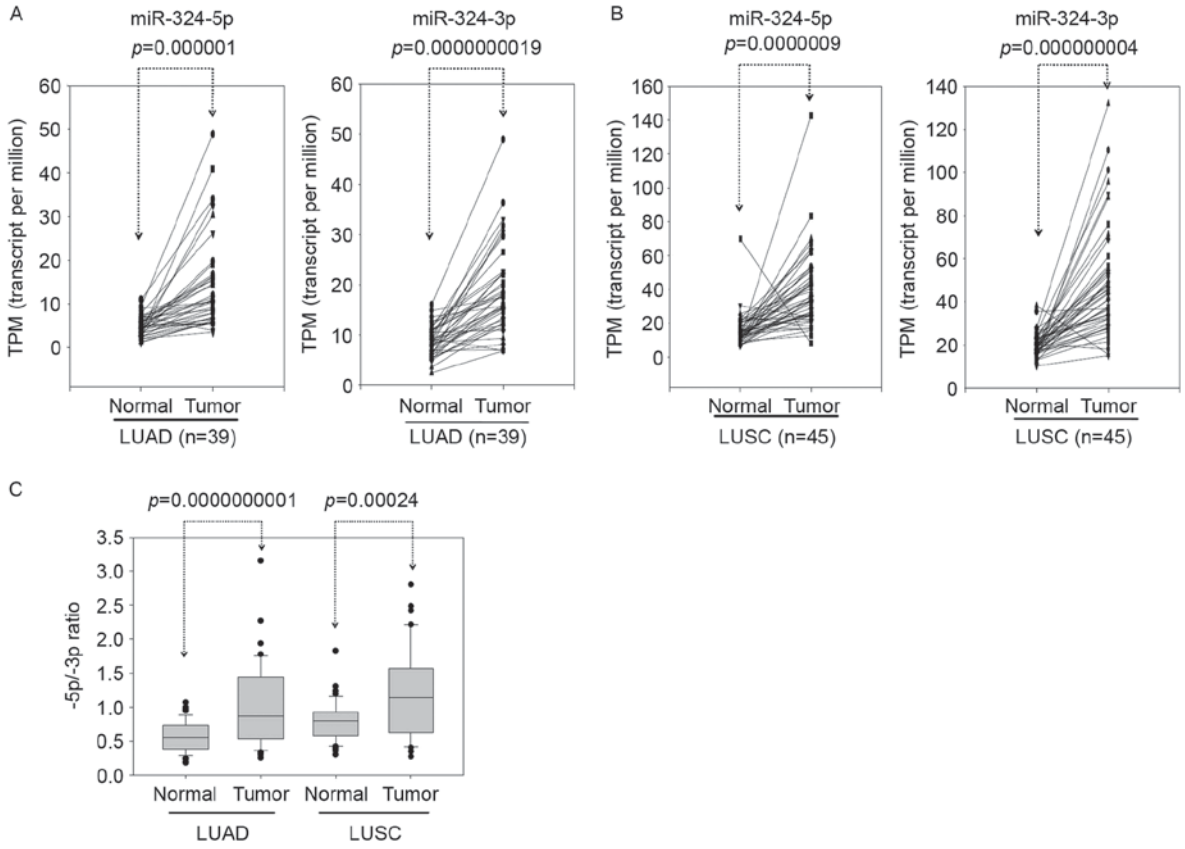


Figure 2. Expression of miR-324-5p and -3p in LUAD, LUSC, and corresponding adjacent normal tissues. (A) Expression of miR-324-5p (left panel) and miR-324-3p (right panel) was examined in LUAD and corresponding adjacent normal tissues obtained from 39 patients. (B) Expression of miR-324-5p (left panel) and miR-324-3p (right panel) was examined in LUSC and corresponding adjacent normal tissues obtained from 45 patients. (C) The miR-324-5p:-3p ratio in LUAD and LUSC was obtained by analyzing The Cancer Genome Atlas. Data were analyzed using the Student's t test. miR, microRNA; LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma.

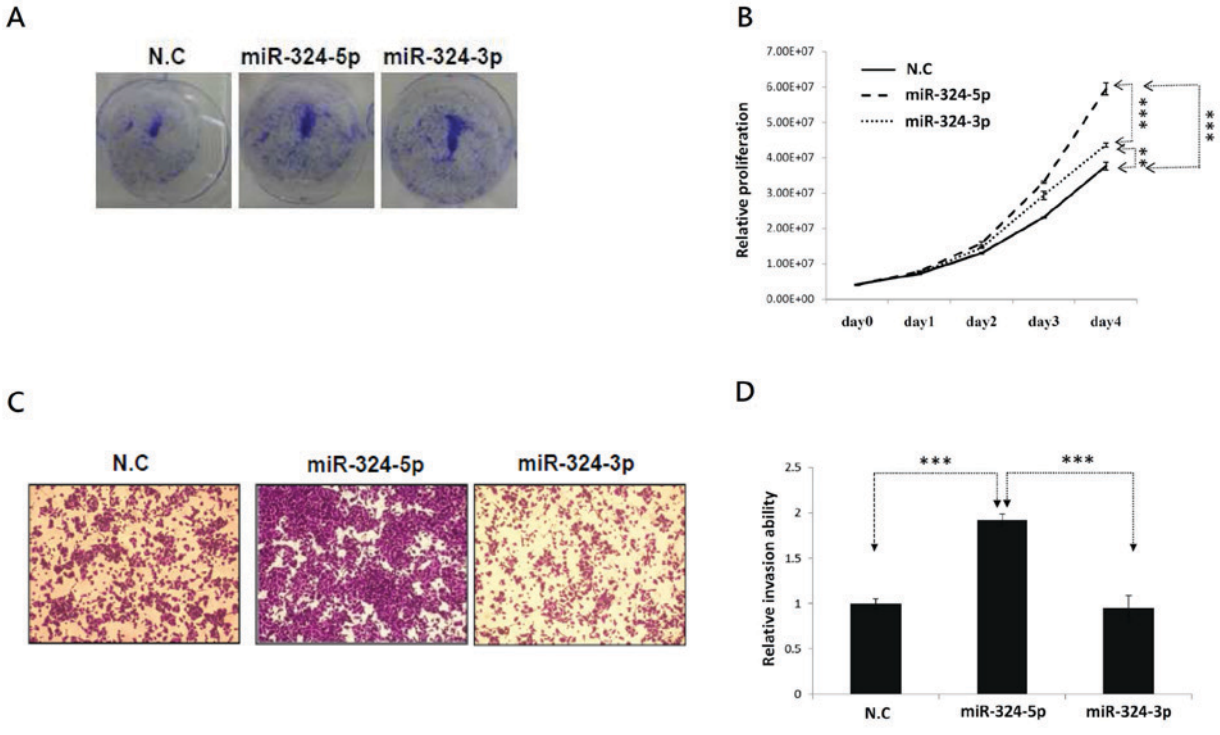
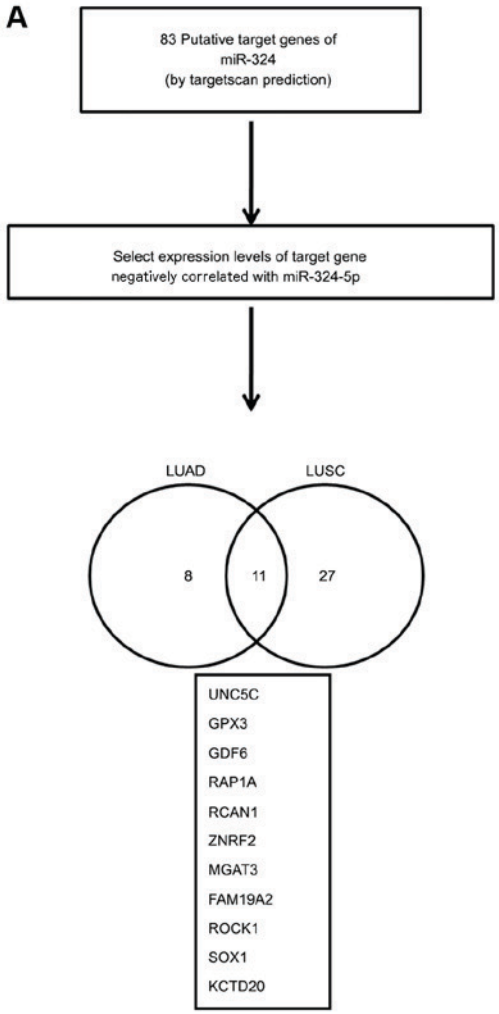


Figure 3. Biological functions of miR-324-5p and -3p arms, as examined in A549 cells. (A) Images of a representative colony formation experiment ($4x10^3$) are presented. (B) Following miR-324-5p or -3p mimic transfection, cell proliferation was measured using the CellTiter-Glo® One Solution assay at 0, 1, 2, 3, and 4 days compared with the N.C. (C) The invasion of the cells was examined using a Transwell assay. (D) The invading cells were counted in three fields under a phase-contrast microscope. **P<0.01 and ***P<0.001, with comparisons indicated by lines. miR, microRNA; N.C, negative scramble control.



B

Gene name	N mean	T mean	P value	T/N	Heatmap
UNC5C	45.69	26.91	0.003	0.59	
GPX3	19,810.00	5,150.00	<0.001	0.26	
RAP1A	2,184.00	1,491.00	<0.001	0.68	
RCAN1	2,613.00	772.5	<0.001	0.3	
ZNRF2	458.6	661.6	<0.001	1.44	
MGAT3	1,451.00	188	<0.001	0.13	
GDF6	11.8	11.07	0.9	0.94	
FAM19A2	19.91	9.528	<0.001	0.48	
ROCK1	1,831.00	1,298.00	<0.001	0.71	
SOX1	0.1401	5.069	0.004	36.18	
KCTD20	1,622.00	1,492.00	0.073	0.92	

N LUAD

C

Gene name	N mean	T mean	P value	T/N	Heatmap
UNC5C	62.45	24.88	<0.001	0.34	
GPX3	23,180.00	2,491.00	<0.001	0.11	
RAP1A	2,082.00	1,197.00	<0.001	0.57	
RCAN1	2,749.00	1,145.00	<0.001	0.42	
ZNRF2	419.3	631.4	<0.001	1.51	
MGAT3	1,126.00	333.8	<0.001	0.3	
GDF6	19.27	21.47	0.83	1.11	
FAM19A2	18.02	14.73	0.5304	0.82	
ROCK1	1,826.00	1,269.00	<0.001	0.69	
SOX1	0.0697	21.96	0.0173	315.06	
KCTD20	1,853.00	1,565.00	0.0016	0.84	

N LUSC

Figure 4. Identification of putative target genes of miR-324-5p using a bioinformatics approach. (A) Workflow for identifying putative target genes of miR-324-5p in lung cancer. A total of 83 target genes of miR-324-5p were identified using the TargetScan tool. The correlations between target genes and miR-324-5p were examined in LUAD and LUSC via TCG A analysis. The Venn diagrams present the 11 putative target genes with expression levels negatively correlated with miR-324-5p expression levels in LUAD and LUSC. The expression levels of potential targets were analysed in (B) LUAD (26 normal and 227 tumor tissues) and (C) LUSC (24 normal and 234 tumor tissues) from TCGA. miR, microRNA; TCGA, The Cancer Genome Atlas; UNC5C, unc-5 netrin receptor C; GPX3, glutathione peroxidase 3; RAP1A, RAP1A, member of RAS oncogene family; RCAN1, regulator of calcineurin 1; ZNRF2, zinc and ring finger 2; MGAT3, mannosyl (β-1,4-) glycoprotein β-1,4-N-acetylglucosaminyltransferase; GDF6, growth differentiation factor 6; FAM19A2, family with sequence similarity 19 member A2, C-C motif chemokine like; ROCK1, Rho associated coiled-coil containing protein kinase 1; SOX1, SRY-box 1; KCTD20, potassium channel tetramerization domain containing 20.

in lung cancer. However, the underlying mechanisms require further clarification in the future.

Discussion

An identical pre-miRNA structure consists of two arms (5p and 3p) and is selected by the RNA-induced silencing complex to generate two mature miRNAs in the cell (8,9,40). Typically, the arm is selected on the basis of hydrogen bond theory. However, a number of previous studies have reported that the arm selection preference of certain miRNAs may vary in different types of cell or during carcinogenesis (20,21,27,41). In humans, significant changes have been reported in the arm selection preference of certain miRNAs between normal and tumor tissues, including gastric cancer, hepatocellular carcinoma, and breast cancer (20,21,27,41). Our group identified significant changes in the arm selection preference of 17 candidate miRNAs in breast cancer compared with adjacent normal tissues by comprehensively analyzing the Sequence Read Archive database (27). Among these, miR-324 arm selection was frequently altered in human cancer, including breast cancer, colon cancer, lung cancer and bladder cancer (28). The arm selection preference of miR-324 is a complex mechanism that modulates its biological function. Through TCGA analysis, miR-324-5p and miR-324-3p expression was frequently observed to be abnormal in human cancer (28). Microarray data have demonstrated that miR-324-5p expression is upregulated in lung cancer, melanoma, hepatocellular carcinoma and acute myeloid leukemia but downregulated in medulloblastoma (42-46). These results consistently indicate the significant upregulation of miR-324-5p in lung cancer.

miR-324-5p and miR-324-3p sequences differ considerably, suggesting that miR-324-5p and -3p serve distinct functions through targeting different protein-coding genes in different types of cancer. Tsai *et al* (27) reported that miR-324-5p and -3p suppressed cell proliferation and invasion in breast cancer,

but only miR-324-5p inhibited cell proliferation and motility in colon cancer (28). An *et al* (39) reported that miR-324-5p suppressed the invasion of hepatocellular carcinoma cells by silencing ETS proto-oncogene 1, transcription factor and Sp1 transcription factor expression. The ectopic expression of miR-324-5p inhibited glioma cell proliferation by suppressing GLI family zinc finger 1 expression (47). Until now, previous studies have primarily focused on the function of miR-324-5p in human types of cancer, while the function of the passenger arm, miR-324-3p, remains unclear. Li *et al* (41) reported that miR-324-3p suppressed the growth of nasopharyngeal carcinoma cells and promoted apoptosis by targeting Smad family member 7. Tsai *et al* (27) reported that miR-324-3p silenced the growth and motility of breast and colon cancer cells. In the present study, the results revealed that miR-324-5p and -3p promoted the proliferation of lung cancer cells, but only miR-324-5p accelerated the invasion of lung cancer cells. The present study is the first to report variation in the arm selection preference of miRNA-#-5p and miR-#-3p in lung cancer compared with healthy tissue. The results provide a novel insight into the arm selection preference of miRNAs, suggesting that this may be a mechanism that modulates their function and further complicates their regulatory network in human types of cancer.

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