MicroRNA-324 in Human Cancer: *miR-324-5p* and *miR-324-3p* Have Distinct Biological Functions in Human Cancer

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Abstract. *MicroRNAs (miRNAs) are small non-coding RNAs that have crucial regulatory functions in carcinogenesis. miR-324-5p and miR-324-3p are generated from the same hairpin RNA structure, however, both are diverse in their direct target genes and expression levels. We report that expression of miR-324-5p and -3p was frequently observed to be either up-regulated or down-regulated, and the selection preference of miR-324 for 5p and 3p arms significantly varied in various types of human cancer. Overexpression of miR-324-5p or -3p suppressed growth and invasion of breast cancer cells. Overexpression of miR-324-5p reduced the growth and invasive abilities of colorectal cancer cells, whereas miR-324-3p suppressed colorectal cancer cell invasion but did not influence cell growth. We conclude that miR-324-5p and miR-324-3p might have distinct biological functions, further complicating the regulatory network in human cancer. Therefore, the arm selection preference of miR-324 may be a method for modulating its function.*

MicroRNAs (miRNAs) are small RNAs with critical functions in several physiological processes (1). Mature miRNAs are produced from primary transcripts (pri-miRNAs) via continuous maturation steps. A Drosha-processed pri-miRNA precursor miRNA (pre-miRNA) structure is composed of 5p and 3p arms and a terminal loop. After being transported to the cytoplasm by exportin 5, the stem-loop structure of pre-miRNAs is divided by DICER to release the terminal loop and 5p-3p duplex. Finally, the -5p or -3p arm of the miRNA is selectively loaded onto the RNA-induced silencing complex according to the hydrogen bond theory (2-4). Thus, an identical pre-miRNA structure generates two mature miRNAs, miR-#-5p and miR-#-3p, which might have distinct functions in cancer progression. miRNA exerts its biological functions by targeting the 3'-untranslated region of protein-coding genes to degrade messenger RNA or inhibit protein translation. The selection preference of miRNA for 5p and 3p arms may vary in different tissues, developmental stages, and species and during cancer progression (5-13). Previous studies have demonstrated that the target-mediated miRNA protection hypothesis participates in the arm selection preference of miRNA (14, 15).

According to the microRNA database (miRBase Release 21; <http://www.mirbase.org/>), miR-324-5p and -3p arms show equal expression levels in human cells. Previous studies have reported that miR-324-3p has a tumour-suppressor function in nasopharyngeal carcinoma and can be an early diagnostic or prognostic biomarker for hepatocellular carcinoma (HCC)

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and colorectal cancer (CRC) (16-18). In addition, miR-324-5p has been shown to suppress glioma and HCC cell growth (19, 20). We previously reported that the selection preference of miR-324 for 5p and 3p arms significantly varied in breast cancer compared to that in adjacent normal tissues analyzed using next-generation sequencing data from the Sequence Read Archive database (8). However, the related biological functions of miR-324-5p and -3p require clarification in human cancer.

In this work, high-throughput sequencing data were downloaded from The Cancer Genome Atlas (TCGA); miR-324-5p and -3p expression levels in various types of human cancer were comprehensively analysed. Furthermore, the roles of miR-324-5p and -3p in the growth and motility of breast cancer and CRC cells were determined.

Materials and Methods

Data on miR-324 expression from TCGA. All level-3 expression data of 15 cancer types were downloaded from TCGA (<https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm>). We obtained the level-3 miRNA data of 5,808 cancer tissues and 641 corresponding adjacent normal tissues.

Cell lines. Five human breast cancer cell lines (H184B5F5/M10, MCF7, MDA-MB-231, AU-565, and MDA-MB-361) and five CRC cell lines (DLD-1, HCT116, SW480, LoVo and colo205) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were maintained in Dulbecco's modified Eagle's medium or RPMI (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (Hyclone, Logan, UT, USA) and penicillin-streptomycin (100 U/ml penicillin and 100 µg/ml streptomycin; Sigma Aldrich, St. Louis, MO, USA).

Clinical samples. A total of 54 breast cancer and 29 corresponding adjacent normal breast tissue samples were collected from patients with breast cancer who underwent surgery at the Department of Surgery, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan. In addition, 48 paired tumour and adjacent normal mucosa samples were obtained from patients with CRC who underwent surgery at the Department of Surgery, Veterans General Hospital, Taipei, Taiwan. Our study protocol was independently reviewed and approved by the Institutional Review Boards of Kaohsiung Veterans General Hospital and Taipei Veterans General Hospital (IRB approval number: VGHKS12-CT12-16 and 2013-04-017AC).

RNA extraction. Total RNA was extracted from cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the instruction manual. Briefly, the samples were homogenised in 1 ml of TRIzol reagent and mixed with 0.2 ml of chloroform for protein extraction, and RNA was precipitated by adding 0.5 ml of isopropanol. The concentration, purity, and amount of the total RNA were determined using a Nanodrop 1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA).

miR-324-5p and -3p overexpression after mimic transfection. CRC or breast cancer cells were seeded in a 25T flask at a density of 1×10^6 cells/ml, and these cells were transfected with 10 nM miR-

324-5p and miR-324-3p mimics or a scrambled sequence as a negative control (GenDiscovery Biotechnology Inc., Taipei, Taiwan) by using Lipofectamine RNAiMAX reagent (Invitrogen). The transfected cells were harvested 24 h later, and the expression was examined through stem-loop reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR).

Stem-loop RT-qPCR. A total of 1 µg of total RNA was reverse transcribed in a stem-loop reverse transcription reaction by using RT primers and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's manual. The reaction was performed under the following incubation conditions: 30 min at 16°C, followed by 50 cycles of 20°C for 30 s, 42°C for 30 s, and 50°C for 1 s. The enzyme was subsequently inactivated by incubation at 85°C for 5 min. Real-time PCR was performed using miR-324-5p- and miR-324-3p-specific forward primers and a universal reverse primer. The reaction was performed at 94°C for 10 min, followed by 40 cycles of 94°C for 15 s and 60°C for 32 s. Gene expression was detected using the SYBR Green I assay (Applied Biosystems, Foster City, CA, USA), and miR-324-5p or miR-324-3p expression was normalised to U6 expression. The following primer sequences were used to examine miRNAs: miR-324-5p- reverse transcript (RT) primer: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACACCAAT-3', miR-324-5p-gene specific forward (GSF): 5'-CGGCGGCGCATCCCCCTAGGGCAT-3'; miR-324-3p-RT: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCAGCAGC-3', miR-324-3p-GSF: 5'-CGGCGGACTGCCCCAGGTGC-3'; universal reverse: 5'-CTGGTGTCTGGAGTCGGCAATTC-3'; U6-F: 5'-CTCGCTTCGCGAGCACA-3', U6-R: 5'-AACGCTTCACGAATTTGCGT-3'.

Cell proliferation and colony formation assay. In the clonogenic assay, cells (2×10^3) were seeded in six-well plates and transfected with 10 nM miR-324-5p and miR-324-3p mimics or a negative control. The cells were incubated in a CO₂ incubator at 37°C for 2 weeks until the formation of colonies with substantial sizes. The medium was removed, cells were fixed in 1 ml of 10% formaldehyde, and plates were incubated at room temperature for 2 min. After removing the formaldehyde solution, 1 ml of crystal violet was added, and the plates were incubated at room temperature for 2 h. The crystal violet solution was then removed, and the plates were rinsed and air-dried at room temperature. Subsequently, 1 ml of 10% acetic acid was added to each well to dissolve the crystal violet. The absorbance values of individual wells were determined at 595 nm using a Multiskan FC (Thermo Fisher Scientific Inc, Wilmington, ED, USA). All experiments were performed in triplicate.

Cell invasion assays. The invasion ability of the cells were assessed *in vitro* by using a transwell assay. Briefly, the cells (4.5×10^5) were resuspended in 2% foetal bovine serum and added to the uppermost chambers of the transwells (Falcon, Corning Incorporated, New York, NY, USA) with a Matrigel coating (BD Biosciences, Bedford, MA, USA) for the invasion assays. The chambers were incubated in a CO₂ incubator at 37°C for 12 or 24 h; the remaining cells in the upper chamber were removed using cotton swabs, and the cells on the undersurface of the transwells were fixed in 10% formaldehyde. The cells were stained with crystal violet, and invading cancer cells in three fields were enumerated under a phase-contrast microscope. All experiments were performed in triplicate.

Figure 1. miR-324-3p (A) and miR-324-5p (B) expression was determined in various cancer types. The Cancer Genome Atlas (TCGA) database was used to retrieve the expression data of miR-324-3p and miR-324-5p in the following: Bladder urothelial carcinoma (BLCA), 19 normal (N) and 416 tumour (T) samples; breast invasive carcinoma (BRCA), 87 N and 778 T; colorectal carcinoma (CRC), 11 N and 314 T; esophageal carcinoma (ESCA), 13 N and 187 T; head and neck squamous cell carcinoma (HNSC), 44 N and 488 T; kidney chromophobe (KICH), 44 N and 67 T; KIRC, 71 N and 261 T; kidney renal clear cell carcinoma (KIRP), 34 N and 292 T; liver hepatocellular carcinoma (LIHC), 50 N and 375 T; lung adenocarcinoma (LUAD), 46 N and 458 T; lung squamous cell carcinoma (LUSC), 45 N and 342 T; prostate adenocarcinoma (PRAD), 52 N and 499 T; stomach adenocarcinoma (STAD), 45 N and 399 T; thyroid carcinoma (THAD), 59 N and 514 T; and uterine corpus endometrial carcinoma (UCEC), 21 N and 418 T. The relative expressions of miR-324-3p and miR-324-5p were assessed in the aforementioned human cancer types and compared to the expression in their corresponding adjacent normal tissues. C: The ratios of 5p and 3p arm selection preferences of miR-324 significantly different between human cancer and the corresponding adjacent normal tissues. Each box represents the quartile distribution (25-75%) range with the median indicated with a black horizontal line. The 95% range including individual outlier samples is also displayed with black dot. The miRNA expression levels were evaluated and are presented as transcripts per million (TPM). Data were analyzed using Student's t-test. Significantly different at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. NS: Not significantly different, $p \geq 0.05$.

Figure 2. 3p and 5p arm selection preference of miRNAs in breast cancer and colorectal cancer (CRC) cells and clinical samples. The 3p and 5p arm selection preferences of miR-324 in five breast cancer (A) and five CRC (E) cell lines. The expression levels of miR-324-3p and miR-324-5p were examined through stem-loop reverse transcription-real-time polymerase chain reaction in 54 breast cancer tissues and 29 corresponding adjacent normal tissues (B and C, respectively) and in CRC tissues and the corresponding adjacent normal tissues isolated from 48 patients. (F and G, respectively). The 5p and 3p arm selection preferences of miR-324 in breast cancer (D) and CRC (H) tissues were compared to that in the corresponding adjacent normal tissues. Each box represents the quartile distribution (25-75%) range with the median indicated with a black horizontal line. The 95% range including individual outlier samples is also displayed with black dot. The expression levels of miR-324 were normalized to that of U6 ($\Delta Ct = \text{target miR-324 Ct} - \text{U6 Ct}$). Data were analysed using Student's *t*-test. Significantly different at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Statistical analysis. miR-324 expression levels in various cancer types were compared with those in corresponding adjacent normal tissues from the TCGA database by using Student *t*-tests. All experimental data of cell growth, and invasion were obtained in triplicate. Histograms present the mean values, and error bars indicate the standard deviations. These data were analysed using Student *t*-tests. A value of $p < 0.05$ was considered statistically significant.

Results

Differences in miR-324-5p and -3p expression in various types of human cancer. We examined the expression levels of mature miR-324-5p and miR-324-3p in different human cancer types by analysing TCGA data. A total of 6,449 small RNA transcriptome profiles of 15 human cancer types were downloaded from TCGA, namely 5,808 cancer tissues and 641 corresponding adjacent normal tissues. As shown in

Figure 1A, miR-324-3p expression was significantly increased in bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), esophageal carcinoma (ESCA), head and neck squamous cell carcinoma (HNSC), kidney renal papillary cell carcinoma (KIRP), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), stomach adenocarcinoma (STAD), and uterine corpus endometrial carcinoma (UCEC) but decreased in CRC and prostate adenocarcinoma (PRAD). Furthermore, miR-324-5p expression was significantly up-regulated in BLCA, BRCA, ESCA, HNSC, LIHC, lung adenocarcinoma (LUAD), LUSC, PRAD, STAD, and UCEC but down-regulated in CRC, kidney chromophobe (KICH), and KIRC (Figure 1B). Our data revealed the simultaneous increase of miR-324-5p and miR-324-3p expression in BLCA, BRCA, ESCA, HNSC, LIHC, LUAD, LUSC, STAD, and UCEC and decrease in CRC. An interesting finding

Figure 3. Ectopic overexpression of miR-324-3p and miR-324-5p influenced growth of human cancer cells. The colony formations of MCF7 (A), MDA-MB-231 (B), SW480 (C), and HCT116 (D) cells were examined after miR-324-3p and miR-324-5p mimic transfection for 2 weeks. The cell images from a representative experiment are shown (upper panels), and the data were quantified (bottom panels). Data are reported for cell growth in miR-324-3p- and miR-324-5p-overexpressing cells compared to control cells (mean±SD). Data were analyzed using Student's *t*-test. Significantly different at **p*<0.05, ***p*<0.01, and ****p*<0.001.

revealed that only *miR-324-5p* (*p*<0.001) was significantly down-regulated in KICH and KIRC but *miR-324-3p* was not significantly changed in KICH and KIRC compared to the corresponding adjacent normal tissues. A contrasting expression pattern of *miR-324-5p* and *miR-324-3p* was observed in PRAD. Although mature *miR-324-5p* and *miR-324-3p* were transcribed from an identical pri-mir-324, the arm selection preference of miR-324 different in different human cancer types. As shown in Figure 1C, the *miR-324-5p:miR-324-3p* selection ratio was significantly increased in BLCA, CRC, HNSC, LUAD, LUSC, and PRAD. Conversely, the ratio was significantly decreased in BRAC,

KICH, KIRC, and KIRP compared to their corresponding adjacent normal tissues; no differences were observed in ESCA, LIHC, STAD, thyroid carcinoma (THCA), and UCEC. Our data revealed that *miR-324-5p* and *miR-324-3p* might play dual roles in different cancer types, and miR-324 showed different arm selection preferences in human cancer.

Determining the miR-324 arm selection preference in human breast and CRC cell lines. According to the TCGA database, *miR-324-5p* and *miR-324-3p* expression was frequently dysregulated in several human cancer types. We used an experimental approach and further examined *miR-324-5p* and

Figure 4. Ectopic expression of miR-324-5p and miR-324-3p suppresses invasion of breast cancer and colorectal cancer cells. The invasive ability was assessed using transwell assays in MDA-MB-231 (A and B) and HCT116 (C and D) cells. After miR-324-5p and miR-324-3p mimic transfection for 12 h, the cells were subjected to transwell assays and incubated at 37°C for 12 h. The cells that invaded the outer surface of the chamber were enumerated. The cells were subsequently stained with crystal violet, and the invading cells were enumerated by counting three fields under a phase-contrast microscope. Data are reported as invading cells under miR-324-5p and miR-324-3p overexpression compared with those under the control condition (mean±SD). Data were analysed using Student's *t*-test. Significantly different at **p*<0.05, ***p*<0.01, and ****p*<0.001.

miR-324-3p expression in human breast cancer and CRC cell lines and clinical tissues. Total RNAs were extracted from the five breast cancer cell lines H184B5F5/M10, MCF7, MDA-MB-231, AU-565, and MDA-MB-361 and five CRC cell lines DLD-1, HCT116, SW480, LoVo and colo205; subsequently, miR-324-5p and miR-324-3p expression was examined through stem-loop RT-qPCR. As shown in Figures 2A and E, the arm selection preference of miR-324 varied in breast cancer and CRC cell lines.

We determined miR-324-5p and miR-324-3p expression in breast cancer and CRC and revealed that miR-324-3p expression in breast cancer and CRC was significantly down-regulated compared with that in corresponding adjacent normal tissues (Figures 2B and F). miR-324-5p was significantly up-regulated in breast cancer (*p*<0.05) but was not significantly changed in CRC (*p*=0.74) (Figures 2C and G). The miR-324-5p:miR-324-3p selection ratios in breast cancer and CRC were significantly increased compared with those in adjacent normal tissues (Figures 2D and H).

miR-324-5p and miR-324-3p modulate cancer cell growth and motility. To understand the individual biological functions of miR-324-5p and miR-324-3p in human cancer cells, breast cancer and CRC cells were transfected with miR-324-5p or

miR-324-3p mimics or the appropriate scrambled sequence controls by using Lipofectamine RNAiMAX reagent. The effects of miR-324-5p or miR-324-3p overexpression on cell growth were analysed using clonogenic assays. As shown in Figures 3A and B, miR-324-5p and miR-324-3p overexpression clearly suppressed the colony formation of breast cancer cells compared with that of the control cells (*p*<0.001). In CRC cells, miR-324-5p clearly inhibited cell growth but ectopic miR-324-3p did not influence SW480 and HCT116 cell growth (Figures 3C and D).

We further examined the effects of miR-324-5p and miR-324-3p on human cancer invasion ability by using transwell assays. As shown in Figure 4, both miR-324-5p and miR-324-3p significantly reduced MDA-MB-231 and HCT116 cell invasion compared with the control. However, the mechanisms of miR-324 in suppressing cell growth and invasion remain unclear and require additional studies.

Discussion

A hairpin RNA structure can generate two mature miRNAs by the microRNA maturation process. Li *et al.* reported that both miR-193a-5p and miR-324-3p synergistically suppressed lung cancer cell metastasis by co-modulating

the Erb-B2 receptor tyrosine kinase 4 (ERBB4)/phosphoinositide-3-kinase regulatory subunit 3 (PIK3R3)/mechanistic target of rapamycin (mTOR)/ribosomal protein S6 kinase 2 (*S6K2*) signalling pathway (21). Our previous study indicated that *miR-193a-3p* suppressed breast cancer cell growth, migration and invasion, whereas *miR-193a-5p* suppressed cell growth but did not influence cell motility (14). Almeida *et al.* reported that the ectopic expressions of different arms of miR-28 have different effects on CRC cell proliferation and migration (22). Therefore, the 5p and 3p arms of miRNA might act similarly or have contrasting biological functions depending on their target genes. Two mature miR-324, miR-324-5p and miR-324-3p, were derived from pre-miR-324 during the maturation process. According to the hydrogen bonding theory, the miR-324-5p:miR-324-3p ratio should be consistent in different human cancer types. However, we obtained interesting data showing significantly different arm selection preferences of miR-324 in human cancer tissues and the corresponding adjacent normal tissues (Figure 1C). This finding is in concordance with our previous findings that arm selection preferences of some miRNAs varied in different tissues, developmental stages, species, and cancer types (7-10, 23).

Previous studies analysing microarray data have revealed *miR-324-5p* up-regulation in lung cancer, malignant melanoma, HCC, and acute myeloid leukaemia but down-regulation in medulloblastoma (24-28). In the present study, by analyzing TCGA data, we provide a consistent result that *miR-324-5p* was significantly up-regulated in LUAD and LUSC (Figure 1B). In addition, we performed comprehensive analysis of *miR-324-5p* and *miR-324-3p* expression levels, revealing that both were frequently either markedly up-regulated or down-regulated in different types of human cancer (Figure 1). However, the expression levels of *miR-324-5p* in CRC and of *miR-324-3p* in breast cancer were inconsistent with TCGA data (Figure 1 and 2). This inconsistency might have resulted from different ethnicities.

Until now, few studies have investigated the biological functions of miR-324 in human cancer progression. Only one study reported that *miR-324-3p* has a tumour-suppression function, suppresses nasopharyngeal carcinoma cell growth, and increases apoptosis through SMAD family member 7 (*SMAD7*) silencing (18). Xu *et al.* reported that *miR-324-5p* can inhibit the proliferation of glioma cells through GLI family zinc finger 1 (*GLI1*) silencing (19). In addition, *miR-324-5p* down-regulation and ectopic miR-324-5p expression can lead to the reduction of migration and invasion by modulating matrix metalloproteinase 2 (*MMP2*), *MMP9*, ETS proto-oncogene 1 (*ETS1*), and Sp1 transcription factor (*SP1*) gene expression in HCC (20). Our findings indicated that the ectopic expression of *miR-324-5p* or *miR-324-3p* can

suppress CRC cell invasion *in vitro*, whereas *miR-324-3p* cannot influence CRC growth (Figure 3 and 4). Therefore, *miR-324-5p* and *miR-324-3p* might play distinct roles in different cancer types. This phenomenon depends on their target genes in different cancer types.

Our findings suggest that studies of the biological functions of miR-324 that use precursor molecules may yield confusing results because the transcription of *miR-324-5p* and *miR-324-3p* has distinct biological effects, further complicating the regulatory network in human cancer. Furthermore, the arm selection preference of miR-324 may be a way to modulate miR-324 function.

Competing Financial Interests

The Authors declare that they have no competing interests.

Acknowledgements

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