MicroRNA expression profiles associated with the metastatic ability of MDA-MB-231 breast cancer cells

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Abstract. Breast cancer is an important worldwide public health concern. The incidence rate of breast cancer increases every year. The primary cause of death is metastasis, a process by which cancer cells spread from a primary site to secondary organs. MicroRNAs (miRs/miRNAs) are small non-coding RNAs that control gene expression at the post-transcriptional level. Dysregulation of certain miRNAs is involved in carcinogenesis, cancer cell proliferation and metastasis. Therefore, the present study assessed miRNAs associated with breast cancer metastasis using two breast cancer cell lines, the low-metastatic MCF-7 and the highly metastatic MDA-MB-231. miRNA array analysis of both cell lines indicated that 46 miRNAs were differentially expressed when compared between the two cell lines. A total of 16 miRNAs were upregulated in MDA-MB-231 compared with MCF-7 cells, which suggested that their expression levels may be associated with the highly invasive phenotype of MDA-MB-231 cells. Among these miRNAs, miR-222-3p was selected for further study and its expression was confirmed by reverse transcription-quantitative PCR (RT-qPCR). Under both non-adherent and adherent culture conditions, the expression levels of miR-222-3p in the MDA-MB-231 cell line were higher than those noted in the MCF-7 cell line under the same conditions. Suppression of endogenous miR-222-3p expression in MDA-MB-231 cells using a miR-222-3p inhibitor resulted in a 20-40% reduction in proliferation, and a ~30% reduction in migration, which suggested that the aggressive phenotype of MDA-MB-231 cells was partly regulated by miR-222-3p. Bioinformatic analysis of miR-222-3p using TargetScan 8.0, miRDB and PicTar identified 25 common mRNA targets, such as cyclin-dependent kinase inhibitor 1B, ADP-ribosylation factor 4, iroquois homeobox 5 and Bcl2 modifying factor. The results of the present study indicated that miR-222-3p was potentially associated with the proliferation and migratory ability of the MDA-MB-231 cell line.

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Abbreviations: miR/miRNA, microRNA; MCF-7, Michigan Cancer Foundation-7; MDA-MB-231, M.D. Anderson-Metastatic Breast 231; TNBC, triple-negative breast cancer; PR, progesterone receptor; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; polyHEMA, poly-2-hydroxyethyl methacrylate; LN, lymph node; cDNA, complementary DNA; RT-qPCR, reverse transcription-quantitative PCR; CDKN1B, cyclin-dependent kinase inhibitor 1B; EIF5A2, eukaryotic translation initiation factor 5A2; IRX5, iroquois homeobox 5; ARF4, ADP-ribosylation factor 4; BMF, Bcl2 modifying factor; ESR1, estrogen receptor 1; has, *Homo sapiens*; TSCC, tongue squamous cell carcinoma; OTSCC, oral tongue squamous cell carcinoma; MMP1, matrix metalloproteinase 1; EOC, epithelial ovarian cancer

Key words: breast cancer, metastasis, miRNA

Introduction

Breast cancer is one of the most common invasive cancers among women and has a high mortality rate. Globally, 2.3 million women were diagnosed with breast cancer in 2020, and 685,000 deaths were reported due to breast cancer (1). Breast cancer is classified into four subtypes based on the expression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). These four subtypes include luminal A ($ER^+/PR^+/HER2^-$), luminal B ($ER^+/PR^+/HER2^+$ or $ER^+/PR^+/HER2^-$), HER2 ($ER^-/PR^-/HER2^+$), and triple negative breast cancer (TNBC) or basal-like ($ER^-/PR^-/HER2^-$) breast cancer. These subtypes possess different abilities to metastasize to the distal organs, with luminal A having the longest survival time followed by luminal B, HER2, and TNBC, respectively (2). Remarkably, TNBC accounts for 10-20% of all cases (3) and is associated with high metastasis, which leads to poor prognosis and high mortality (4). Patients with the TNBC subtype do not respond well to hormonal therapies, such as HER2-targeted therapy (3), which is a significant challenge for the successful treatment of patients with TNBC. Therefore, the development of novel biomarkers for the identification of the TNBC subtype is crucial.

The two breast cancer cell lines, MCF-7 and MDA-MB-231 have been well characterized for their low and high metastatic phenotypes, respectively. The MCF-7 cell line represents the luminal A subtype, while the MDA-MB-231 cell line represents the TNBC subtype (5). The MCF-7 cell line can form only the primary tumor and can be suppressed following treatment by tamoxifen, an ER antagonist, while the MDA-MB-231 cell line can induce metastasis and is resistant to tamoxifen (5).

MicroRNAs (miRs/miRNAs) are small non-coding RNAs (snRNA) 18-22 nucleotides in length, which regulate several biological processes in tumorigenesis including proliferation, stress response, cell adhesion, motility and apoptosis (6). Previous studies have reported aberrant expression of certain miRNAs in TNBC. For example, Fang *et al* (7) reported that miR-21 was upregulated in MDA-MB-468 cells and that it supported proliferation and invasion. Similarly, miR-25-3p has been reported to be overexpressed in TNBC where it promotes proliferation both *in vitro* and *in vivo* (8). Previous studies have also reported that elevated expression of miR-93 (9) and miR-455-3p (10) induced proliferation, invasion and metastasis in TNBC. Downregulation of certain miRNAs has also been reported in TNBC; they have been demonstrated to regulate proliferation, migration and invasion in TNBC (6). These data support the roles of miRNAs in maintaining the invasive phenotype of TNBC and therefore hold promise for their potential application as diagnostic and prognostic biomarkers.

A previous study indicated that miR-222 expression was elevated in breast cancer tissues compared with that of normal tissues, which suggested that it could be a promising biomarker for breast cancer diagnosis (11). miR-222 expression in breast cancer tissues exhibited prognostic significance in lymph node (LN) negative breast cancer, which suggested that it could be used as a differential biomarker between LN negative and positive patients (12). Although miR-222 was highly expressed in breast cancer tissues, it remains unclear whether this miRNA is highly expressed in TNBC. The present study aimed to study miRNAs associated with breast cancer metastasis using two breast cancer cell lines, the low-metastatic MCF-7 cell line and the highly metastatic MDA-MB-231 cell line. The expression of one candidate miRNA, miR-222-3p, was validated. Suppression of miR-222-3p expression was performed to study its roles in MDA-MB-213 cells. The data indicated the crucial roles of miR-222-3p in supporting growth and migration in TNBC.

Materials and methods

Cell culture. The breast cancer MCF-7 (HTB22; American Type Culture Collection) and MDA-MB-231 (HTB26; American Type Culture Collection) cell lines were cultured in DMEM high glucose (HyClone™; Cytiva) supplemented with 10% (v/v) FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin at 37˚C, in the presence of 5% CO_2. For the anoikis resistance assay, 700 μl of 20 mg/ml poly-2-hydroxyethyl methacrylate (polyHEMA) diluted in 95% ethanol was applied to a 6-well plate and dried for 2 days. Breast cancer cells were plated ($5x10^5$ cells/well for MCF-7 and $1x10^6$ cells/well for MDA-MB-231) in a polyHEMA-coated 6-well plate.

RNA extraction, complementary DNA (cDNA) synthesis and miRNA array. RNA was extracted from the adherent and anoikis-resistant MCF-7 and MDA-MB-231 cells using TRIzol® reagent according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 200 ng RNA from each cell line was used to synthesize cDNA using the miRCURY LNA RT Kit (cat. no. 339340; Qiagen, Inc.) according to the manufacturer's protocol. To determine the miRNA expression profiles, an miRNA array was performed using miRCURY LNA miRNA focus panel (cat. no. 339325; Qiagen, Inc.) coated with specific primers for 84 miRNAs associated with cancer. The miRNA target sequences are presented in Table SI. The miRCURY LNA SYBR Green PCR Kit(cat no. 339345; Qiagen, Inc.) was used for qPCR reactions. The 96-well plate of the miRNA array was subjected to PCR using a real-time PCR machine. The PCR cycling conditions were: Initial heat activation at 95˚C for 2 min, and 40 cycles of denaturation at 95˚C for 10 sec and annealing/extension at 56˚C for 60 sec. The relative expression was determined using the $2^{-\Delta\Delta Cq}$ method (13).

RT-qPCR. A candidate miRNA, miR-222-3p, from the miRNA array was assessed using RT-qPCR. RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A total of 100 ng RNA extracted from adherent or anoikis-resistant MCF-7 and MDA-MB-231 cultures was converted to cDNA using a TaqMan® Advanced MicroRNA Synthesis Kit (cat. no. A25576; Applied Biosystems; Thermo Fisher Scientific, Inc.). cDNA synthesis was performed using four steps, including Poly (A) tailing, adaptor ligation, RT and cDNA amplification reactions, according to the manufacturer's protocol. The expression levels of miR-222-3p were determined by qPCR using TaqMan Advanced MicroRNA Assays (miR-222-3p, cat. no. A25576; Applied Biosystems; Thermo Fisher Scientific, Inc.) containing forward and reverse primers and probes in a single tube and TaqMan® Fast Advanced Master Mix (cat. no. 4440038; Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The qPCR thermal conditions were: Uracil-N-glycosylase incubation at 50˚C for 2 min and polymerase activation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing/extension at 60˚C for 60 sec. The relative expression was compared using the $2^{-\Delta\Delta Cq}$ method (13) and U6 snRNA (cat. no. 4427975; Applied Biosystems; Thermo Fisher Scientific, Inc.) was used as the internal control. The miRNA sequences used in this experiment were as follows: Mature miRNA sequence of miR-222-3p, 5'-AGCUACAUCUGGCUACUGGGU-3' (cat. no. A25576; Applied Biosystems; Thermo Fisher Scientific, Inc.); and U6 snRNA control sequence, 5'-GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAACGATACAGAGAAGATTAGCATGGCCCCTGCGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTT-3' (cat. no. 4427975; Applied Biosystems; Thermo Fisher Scientific, Inc.). The forward and reverse primer sequences used in this experiment are proprietary information of Applied Biosystems; Thermo Fisher Scientific, Inc.

Transfection of cells with the miR-222-3p inhibitor. A total of $2.5x10^5$ MDA-MB-231 cells were plated in a 12-well plate and incubated at 37˚C overnight, and 50 nM miR-222-3p

inhibitor (cat. no. 339121; Qiagen, Inc.) or 50 nM negative control (cat. no. 339126; Qiagen, Inc.) was transfected into the cells using HiPerFect Transfection Reagent (cat. no. 301704, Qiagen, Inc.). The transfection was performed according to the manufacturer's protocol (Qiagen, Inc.). miR-222-3p inhibitor or negative control was diluted with 100 μl culture medium without serum. HiperFect transfection reagent (3 μl) was added, followed by mixing by vortexing. The mixtures were incubated at room temperature for 10 min to allow the formation of transfection complexes before addition to the cells. The plate was gently swirled to ensure uniform distribution of the transfection complexes. Then, the cells were incubated with the transfection complexes at 37˚C for 48 h before use in further experiments. The negative control (cat. no. 339126; Qiagen, Inc.) used in this experiment was scramble-miRNA control (negative control A miRCURY LNA miRNA inhibitor control), which demonstrated no hits of >70% homology for any sequence in any organism in the NCBI (https://www.ncbi.nlm.nih.gov/) and miRBase (https://www.mirbase.org/) databases. The sequences of negative control and miR-222-3p inhibitor were 5'-TAACACGTCTATACGCCCA-3' and 5'-CCCAGTAGCCAGATGTAGC-3', respectively. To confirm miR-222-3p inhibition, qPCR was performed at 48 h post-transfection. The relative expression levels of miR-222-3p were compared between the miR-222-3p inhibitor and scramble control transfected cells. The expression levels of miR-222-3p of miRNA inhibitor-transfected cells were relative to those of the scramble control cells. The expression levels of the scramble control cells were set as 100%.

Trypan blue exclusion assay. The transfected cells were collected at 48 h post-transfection and re-plated (100,000 cells/well) into 12-well plates for 5 days. The cells were incubated at 37˚C in a 5% CO_2 incubator and counted at days 1, 2, 3, 4 and 5. At each time point, the cells were trypsinized using 0.25% Trypsin-EDTA (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C for 3 min and resuspended with 900 μl culture media. Cell suspension (100 μl) was mixed with 100 μl 0.4% trypan blue (Gibco; Thermo Fisher Scientific, Inc.) to obtain a 1:2 dilution. The mixture was incubated at room temperature for 5 min to allow the trypan blue to stain the dead cells. Subsequently, the cells were counted using a hemocytometer under a light microscope. The results are presented as the mean cell density (cells/ml) ± SD.

Migration assay. The migration assay was performed using Transwell inserts (6.5 mm diameter, polyvinylpyrrolidone-free polycarbonate filters with 8 μm pore size; Corning, Inc.). A total of $1.2x10^5$ cells were re-suspended in 200 μl serum-free DMEM and plated into the upper chamber of the Transwell insert. A total of 600 μl DMEM supplemented with 10% (v/v) FBS (Gibco; Thermo Fisher Scientific, Inc.) was added to the lower chamber to induce cell migration. Following 6 h of incubation at 37˚C, the migrated cells were fixed using 4% paraformaldehyde in 1X PBS at room temperature for 20 min and stained using 0.5% crystal violet in 25% methanol as previously described (14). The number of migrated cells was counted in 5 different randomly selected fields of view under a light microscope, and the numbers in the miR-222-3p knockdown and scrambled control groups were compared. The data are presented as the mean ± standard deviation (miR-222-3p knockdown cells vs. scrambled control cells).

miRNA target prediction. The mRNA targets of miR-222-3p were predicted using three programs, including TargetScan 8.0 (15), miRDB (16) and PicTar (17). The total context++ score was calculated using TargetScan 8.0 (15,18), and the mRNA targets were ranked from the lowest to the highest total context++ score (Table SII).

Statistical analysis. The results were obtained from two independent experiments, each in duplicate. All values are presented as mean ± standard deviation. The statistical analysis was performed using unpaired Student's t-test, Mann-Whitney test and one-way ANOVA followed by Tukey's multiple comparison test. Statistical analysis was performed using GraphPad Prism version 5.0 software (Dotmatics). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Differential miRNA expression profiles in MCF-7 and MDA-MB-231 cell lines. To determine the miRNA expression profiles associated with the invasive phenotype, an miRNA array was used to assess the MCF-7 and MDA-MB-231 breast cancer cell lines. Due to the different characteristics of the two cell lines, notably the degree of metastatic potential, it was hypothesized that miRNAs involved in metastasis would be highly expressed in MDA-MB-231 cells. A total of 46 miRNAs were demonstrated to be differentially expressed between these two cell lines. A total of 16 miRNAs were upregulated and 30 miRNAs were downregulated in MDA-MB-231 cells compared with MCF-7 cells (Fig. 1). Details of the upregulated and downregulated miRNAs are presented in Tables I and II, respectively.

Expression levels of miR-222-3p are upregulated in MDA-MB-231 breast cancer cells. Based on the miRNA array, there were 16 upregulated miRNAs in highly metastatic MDA-MB-231 cells compared with low-metastatic MCF-7 cells, indicating that these miRNAs may be associated with breast cancer metastasis (Table I). One candidate miRNA, miR-222-3p, was validated in the present study. The miRNA array results indicated that the miRNA expression levels of miR-222-3p were upregulated in the MDA-MB-231 cell line; the levels were ~15-fold higher than those noted in the MCF-7 cell line. Therefore, miR-222-3p may be associated with the invasive phenotype of MDA-MB-231 cells. The miRNA expression levels of miR-222-3p were further assessed in MDA-MB-231 and MCF-7 cell lines using RT-qPCR. The miR-222-3p expression levels in MDA-MB-231 cells were 250-fold higher than those demonstrated in MCF-7 cells (Fig. 2E). The expression levels of miR-222-3p were also determined under anoikis resistance conditions. The anoikis-resistant cells were generated to mimic the phenotype of those cancer cells which survive following detachment from the primary site and remain in the circulation. MCF-7 and MDA-MB-231 cells were cultured in an anti-adhesive polymer (polyHEMA)-coated plate, which resulted in loss of cell attachment. The cells

Table I. Up-regulated miRNAs in the highly invasive MDA-MB-231 breast cancer cell line compared with the non-invasive MCF-7 breast cancer cell line.

miRNA name	Fold change
hsa-miR-100-5p	32.51
hsa-miR-106a-5p	2.19
hsa-miR-125b-5p	18.4
hsa-miR-130a-3p	47.45
hsa-miR-10b-5p	5.06
hsa-miR-146a-5p	42.31
hsa-miR-17-5p	2.11
hsa-miR-18a-5p	2.86
hsa-miR-10a-5p	4.83
hsa-miR-20a-5p	2.19
hsa-miR-221-3p	26.09
hsa-miR-222-3p	15.85
hsa-miR-29a-3p	10.22
hsa-miR-29b-3p	4.50
hsa-miR-29c-3p	6.16
hsa-miR-30c-5p	2.48

hsa, *Homo sapiens*; miR/miRNA, microRNA.

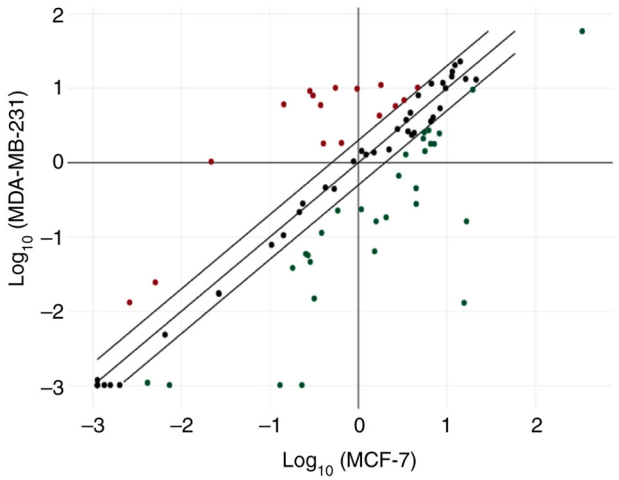

Figure 1. Scatter plot of miRNA expression profiles of the highly invasive breast cancer (MDA-MB-231) and comparison with the non-invasive breast cancer (MCF-7) cell line. miRNAs that showed 2-fold higher or lower expression in MDA-MB-231 cells compared with MCF-7 cells were defined as upregulated miRNAs (red dots) and downregulated miRNAs (green dots), respectively. Black dots indicate miRNAs with unchanged expression levels in MDA-MB-231 cells compared with MCF-7 cells. miRNA, microRNA.

Table II. Down-regulated miRNAs in the highly invasive MDA-MB-231 breast cancer cell line compared with the non-invasive MCF-7 breast cancer cell line.

miRNA name	Fold change
hsa-miR-200a-3p	-23.78
hsa-let-7f-5p	-2.29
hsa-miR-101-3p	-4.57
hsa-miR-106b-5p	-4.02
hsa-miR-126-3p	-3.38
hsa-miR-141-3p	-1,201.53
hsa-miR-145-5p	-3.78
hsa-miR-26a-5p	-2.66
hsa-miR-15a-5p	-2.17
hsa-miR-149-3p	-7.22
hsa-miR-16-5p	-2.04
hsa-miR-182-5p	-11.20
hsa-miR-191-5p	-3.95
hsa-miR-192-5p	-4.31
hsa-miR-148a-3p	-2.58
hsa-miR-194-5p	-6.19
hsa-miR-195-5p	-21.28
hsa-miR-196a-5p	-127.68
hsa-miR-200b-3p	-16.14
hsa-miR-200c-3p	-102.39
hsa-miR-205-5p	-225.92
hsa-miR-21-5p	-5.75
hsa-miR-215-5p	-4.70
hsa-miR-25-3p	-3.66
hsa-miR-26b-5p	-4.28
hsa-miR-34a-5p	-9.75
hsa-miR-7-5p	-9.92
hsa-miR-9-5p	-4.77
hsa-let-7e-5p	-2.56
hsa-miR-93-5p	-3.32

hsa, *Homo sapiens*; miR/miRNA, microRNA.

which survived and proliferated in an anchorage-independent manner were considered to be anoikis-resistant cells (19). The adherent MCF-7 and MDA-MB-231 cells had epithelial-like morphologies. MCF-7 cells formed a monolayer and their shape was dome-like (Fig. 2A) and MDA-MB-231 cells were spindle-shaped (long and thin) (Fig. 2B). The morphologies of MCF-7 and MDA-MB-231 anoikis-resistant cells were round with spheroid shape compared with their parental cell lines (Fig. 2C and D). Following two days of cell culture under anoikis conditions, the anoikis-resistant cells were collected to determine the expression levels of miR-222-3p. Under anoikis-resistance conditions, MDA-MB-231 cells expressed miR-222-3p at a similar level as that noted following cell culture under the adherent conditions (Fig. 2E). The mRNA targets of miR-222-3p were predicted using TargetScan 8.0 (15), miRDB (16) and PicTar (17). TargetScan 8.0, miRDB and PicTar predicted 254, 619, and 177 mRNA targets, respectively. A total of 25 mRNA targets were commonly identified by all three programs (Fig. S1). Certain of these 25 mRNA targets were associated with key proteins involved in cell proliferation and metastasis, such as cyclin-dependent kinase inhibitor 1B (CDKN1B), eukaryotic translation initiation factor 5A2 (EIF5A2), iroquois homeobox 5 (IRX5), ADP-ribosylation factor 4 (ARF4), Bcl2 modifying factor (BMF), and estrogen receptor 1 (ESR1). The 25 common

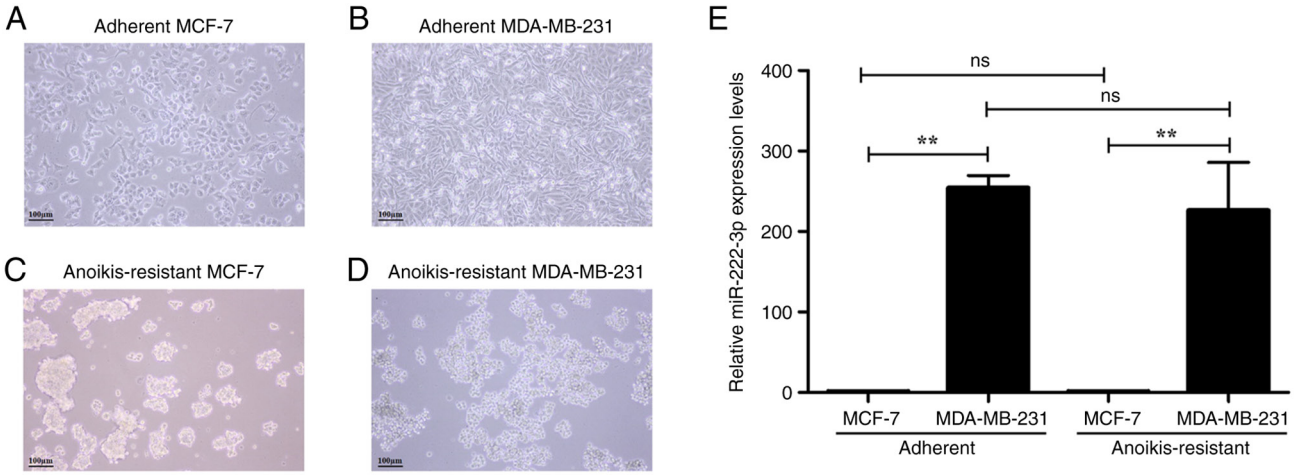

Figure 2. Morphology and miR-222-3p expression of adherent and anoikis-resistant MCF-7 and MDA-MB-231 cells. Representative micrographs of adherent (A) MCF-7 and (B) MDA-MB-231 cells cultured in complete growth medium. The anoikis-resistant (C) MCF-7 and (D) MDA-MB-231 cells were cultured on a poly 2-hydroxyethyl methacrylate plate for two days under the same conditions as the adherent cells. Scale bar, 100 μm. (E) The miRNA expression levels of miR-222-3p in adherent MCF-7 and MDA-MB-231 cells, and anoikis-resistant MCF-7 and MDA-MB-231 cells assessed using reverse transcription-quantitative PCR. The Y-axis Expression levels of miR-222-3p were indicated relative to MCF-7 cells. The results are indicative of two independent experiments. **P<0.01. miR-222-3p expression was significantly higher in MDA-MB-231 cells under both adherent and anoikis-resistant conditions. miR/miRNA, microRNA; ns, not significant.

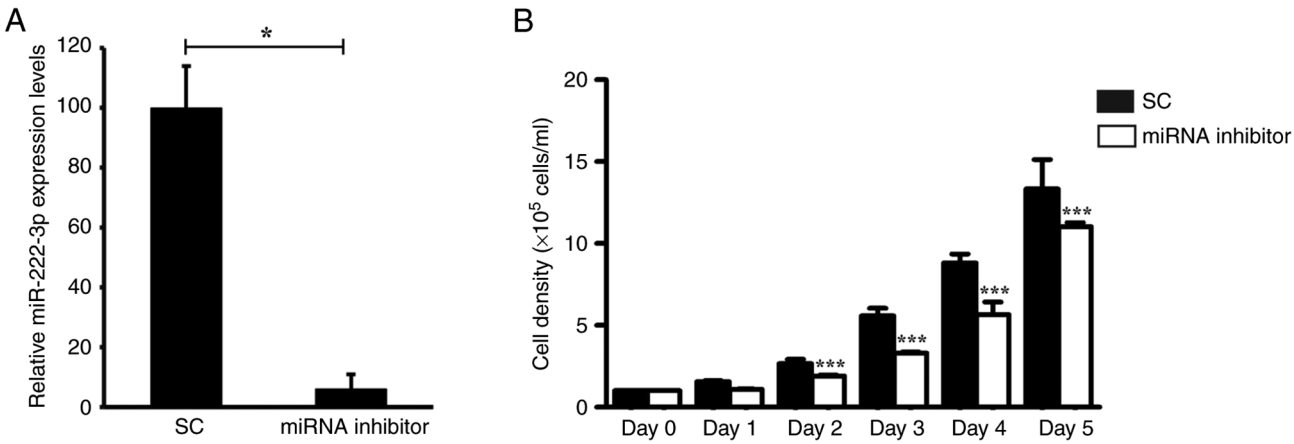

Figure 3. Suppression of miR-222-3p expression reduces proliferation of MDA-MB-231 cells. (A) miR-222-3p expression levels were assessed using reverse transcription-quantitative PCR two days post-transfection. (B) Suppression of miR-222-3p reduced the proliferative ability of MDA-MB-231 cells. MDA-MB-231 cells were transiently transfected with 50 nM miR-222-3p inhibitor or SC siRNA. A total of 2 days post-transfection, the transfected cells were re-plated and counted for 5 days. Data were obtained from two independent experiments, each in duplicate and are presented as mean ± SD. *P<0.05 and ***P<0.001 vs. SC. miR/miRNA, microRNA; SC, scrambled control.

mRNA targets ranked from the lowest to the highest total context++ score calculated using TargetScan 8.0 (15,18) are presented in Table SII.

Suppression of miR-222-3p reduces proliferation in the MDA-MB-231 breast cancer cell line. The miRNA expression levels of miR-222-3p were elevated in the MDA-MB-231 cell line compared with those of the MCF-7 cell line (Fig. 2E). This miRNA may contribute to the proliferative ability of MDA-MB-231 cells. Therefore, the present study assessed whether the suppression of miR-222-3p expression in MDA-MB-231 cells affected their proliferation. Transfection of the cells with the miR-222-3p inhibitor markedly reduced the expression levels of miR-222-3p by 94% (Fig. 3A). At 48 h post-transfection, the proliferative rate of miR-222-3p-knockdown MDA-MB-231 cells was assessed for 5 days. MDA-MB-231 cells transfected with the miR-222-3p inhibitor indicated a significant, 20-40%, reduction in cell viability from day 2 onward (Fig. 3B).

Suppression of miR-222-3p reduces migration in highly metastatic breast cancer MDA-MB-231 cells. As the miRNA expression levels of miR-222-3p were elevated in MDA-MB-231 cells, it was hypothesized that this miRNA may be involved in the migratory ability of this cell line. Endogenous miR-222-3p expression in MDA-MB-231 cells was suppressed using a miR-222-3p inhibitor. At 48 h post-transfection, miR-222-3p-knockdown-MDA-MB-231 cells demonstrated a significant, 30% reduction in migration (Fig. 4).

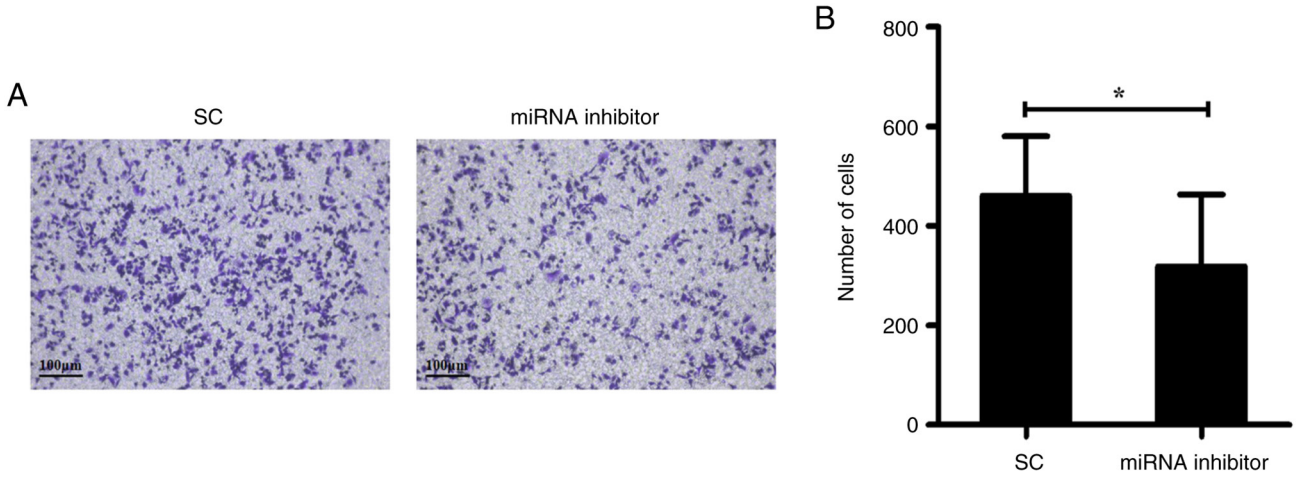

Figure 4. Suppression of miR-222-3p in MDA-MB-231 cells reduces migration of MDA-MB-231 cells. MDA-MB-231 cells were transiently transfected with miR-222 inhibitor or SC. On 2 day-post transfection, the transfected cells were re-plated for use in the migration assay. (A) Representative images of the migrated miR-222-3p knockdown (miR-222 inhibitor) or scrambled control cells (control). (B) The number of migrated cells was counted in 5 different randomly selected fields of view and are presented as mean ± SD. The results were obtained from two independent experiments, each in duplicate. *P<0.05. miR/miRNA, microRNA; SC, scrambled control.

Discussion

miRNAs serve a crucial role in tumorigenesis including proliferation, stress responses, cell adhesion, motility and apoptosis (6). numerous miRNAs have been reported to regulate metastasis in certain types of human cancer (20). In the present study, miRNA expression profiles were assessed in the highly metastatic MDA-MB-231 cells and compared with those in the low-metastatic MCF-7 cells. A total of 16 miRNAs were highly expressed in the highly metastatic MDA-MB-231 cell line; however, their expression levels were low in the MCF-7 cell line, which suggested that these miRNAs may be associated with the aggressive breast cancer phenotype. A total of 30 miRNAs were downregulated in MDA-MB-231 cells compared with MCF-7 cells. From the miRNA expression profiles, these miRNAs could be considered as biomarkers for metastatic breast cancer in the future. However, further studies are required to validate the expression of these candidate miRNAs in breast cancer cell lines and samples from patients with different stages of breast cancer.

miR-221/222 has been previously reported to be an onco-miR or a tumor suppressor miRNA, depending on the cellular context (21). miR-222 has been reported as an onco-miR in gastric cancer, bladder cancer, lung cancer, colorectal cancer, cervical cancer and ovarian cancer; whereas, it has been reported to act as a tumor suppressor miRNA in tongue squamous cell carcinoma (TSCC), colorectal cancer, and ovarian cancer (21). The onco-miRs regulate tumor suppressor genes to promote cancer development, while the tumor suppressor miRNAs target oncogenes to inhibit cancer progression (22). In oral TSCC (OTSCC), miR-222 serves a role as a tumor suppressor miRNA, which inhibits OTSCC cell invasion by regulating matrix metalloproteinase 1 (MMP1) expression. This is mediated by the targeting of MMP1 mRNA and indirectly controlling its gene expression via the targeting of manganese superoxide dismutase 2 (23). miR-222 serves oncogenic roles in gastric cancer. Upregulation of miR-222 induces the cell proliferation and invasion of the gastric cancer cell line SGC7901, whereas suppression of miR-222 reverses these phenotypes via induction of PTEN, a direct target of miR-222 (24). In colorectal cancer, miR-222 directly targets a disintegrin and metalloprotease 17, which is downregulated in multidrug-resistant colorectal cancer cells and increases cancer cell apoptosis (25). In contrast to these reports, miR-222 acts as an onco-miRNA in colorectal cancer cells by the direct targeting of mammalian Ste20-like protein kinase 3 (26), and MIA SH3 domain ER export factor 3 (27) to promote cancer cell migration and invasion.

The association of miR-222 and breast cancer has not been clearly elucidated. A limited number of studies that examined the expression of miR-222 in breast cancer have reported the roles of miR-222 to be coupled with miR-221. Li *et al* (28) reported that the miRNA expression levels of miR-221/222 were elevated in cisplatin-resistant MDA-MB-231 cells and in patients with cisplatin-resistant breast cancer. Suppression of miR-221/222 in MDA-MB-231 cells increased their sensitivity to cisplatin *in vitro* and induced apoptosis, which suggested that the combination of anti-miR-221 and anti-miR-222 produced the synergistic effects noted following cisplatin treatment (28). Therefore, the present study further evaluated the expression and functions of miR-222.

The miRNA array results indicated that miR-222-3p expression was upregulated in MDA-MB-231 compared with MCF-7 cells. The results from RT-qPCR analysis were consistent with the miRNA array results, which indicated that miR-222 was expressed at higher levels in MDA-MB-231 compared with MCF-7 cells. A previous study also reported that miR-222 expression was upregulated in breast cancer tissues compared with those in non-cancerous breast tissues (11). Moreover, miR-222-3p expression was previously reported to be elevated in the sera of patients with breast cancer, which suggested that it could be used as a non-invasive biomarker for human breast cancer. However, this miRNA cannot be used as a biomarker to differentiate between early stage and advanced stage breast cancer because the subjects of the previous study were only from stages II and III of the disease; in addition, the

expression level of miR-222-3p was not significantly different between stage II and stage III tumors (29). The results of the present study, that a higher expression level of miR-222-3p was detected in invasive breast cancer cells, were in line with a previous report that the miR-222 level was elevated in patients with breast cancer with lymphatic metastasis (30). It should be noted that the present study indicated that miR-222-3p expression was significantly higher in MDA-MB-231 cells, which represented the highly metastatic model of breast cancer, compared with the corresponding levels demonstrated in the non-metastatic MCF-7 cells. This indicated the potential of miR-222-3p as a prognostic biomarker for breast cancer metastasis. The present study further examined other functions of this miRNA, including in anoikis-resistance, proliferation and migration in the highly metastatic MDA-MB-231 breast cancer cell line. Inhibition of miR-222-3p in MDA-MB-231 cells suppressed the high proliferation and migration, which are related to breast cancer metastasis. Numerous miRNAs have been reported to promote or suppress anoikis in certain types of cancer. miR-31, miR-220b and miR-200c have been previously reported to enhance anoikis, while miR-181a promotes anoikis-resistance in breast cancer (31). miR-141 has been reported to promote anoikis resistance in ovarian cancer cells and was highly expressed in anchorage-independent ovarian cancer cell lines compared with anchorage-dependent cells (32). However, the results of the present study demonstrated that the expression levels of miR-222-3p in MDA-MB-231 cells under anoikis-resistance condition were not higher than those in the adherent MDA-MB-231 cells. Similarly, the levels of miR-222-3p in anoikis-resistant MCF-7 cells were not higher than those in the adherent MCF-7 cells. These results indicated that miR-222-3p was not associated with the anoikis resistance of breast cancer.

Due to the high miRNA expression level of miR-222 in patients with breast cancer and lymphatic metastasis and in the MDA-MB-231 cell line, this miRNA may serve crucial roles in cell proliferation and motility, which are the pre-requisites for cancer progression. The present study demonstrated that proliferation of miR-222-3p knockdown cells was suppressed by 20-40%, which indicated that this miRNA supported the proliferative ability of MDA-MB-231 cells. Moreover, suppression of miR-222-3p inhibited the migratory ability of MDA-MB-231 cells by ~30%, which indicated that this miRNA induced the migration of MDA-MB-231 cells. The migratory results correlated with those of a previous study on the function of exosomal miR-222, which reported that suppression of miR-222 reduced the migratory ability of MDA-MB-231 cells (30). The study also reported the decreased invasive ability of miR-222 knockdown MDA-MB-231 cells (30). The present study demonstrated the role of miR-222-3p in supporting cancer cell proliferation and to a lesser extent migration. The increased expression of miR-222-3p in the highly metastatic breast cancer cell line may also support other breast cancer phenotypes, such as chemoresistance. According to the functions of this miRNA in cell proliferation and migration in breast cancer, the mRNA targets of miR-222-3p were predicted using bioinformatics analysis (Fig. S1); however, these require validation in future studies. Some of the 25 mRNA targets were reported to be involved in cell proliferation and metastasis in cancer. For example, CDKN1B/p27 has been reported as the target of miR-221 which functions as an oncogenic miRNA in hepatocarcinogenesis by promoting cell proliferation and regulating the expression of cell-cycle inhibitors (33). In addition, the EIF5A2 gene has been reported as a direct target of miR-221-3p and its expression level was decreased in medulloblastoma cell lines (34). Overexpression of miR-221-3p in these cell lines reduced their proliferation (34). The suppressive effect of miR-221-3p on cell proliferation was reported to be alleviated by the restoration of EIF5A2 in miR-221-3p-overexpressing DAOY cells (34). Aberrant expression of IRX5 was previously reported in TSCC tissues and cell lines. Overexpression of IRX5 promoted proliferation, migration, and invasion of TSCC cells, whereas knockdown of IRX5 expression caused the opposite effects (35). BMF was reported as a target regulated by miR-221 using the dual-luciferase reporter gene assay. The inhibition of miR-221 expression significantly increased BMF expression in ovarian cancer SKOV3 cells, accompanied by decreased cell proliferation and increased cell apoptosis (36). ESR1 has been reported as the target of miR-222/221 and its expression levels were reported to be markedly higher in ESR1 negative breast cancer cells. In clinical samples, miR-222 expression was reported only in TNBC, whereas miR-222 was absent in luminal A breast cancer, which indicated that this miRNA acted cooperatively to decrease ERα expression (37). ARF4 is one of the putative targets of miR-221-3p. Overexpression of miR-221-3p inhibited the proliferation and migration of epithelial ovarian cancer (EOC) cells *in vitro*. The negative correlation between ARF4 and miR-221-3p levels was previously reported in EOC specimens, which suggested that the tumor suppressive role of miR-221-3p in EOC may be via the direct targeting of ARF4 (38).

The analysis of additional upregulated miRNAs demonstrated upregulation of miR-100-5p and 146a-5p in MDA-MB-231 cells compared with the corresponding miRNA expression levels demonstrated in MCF-7 cells. Elevated miRNA expression levels of miR-100-5p in post-neoadjuvant chemotherapy samples was reported to be significantly correlated with improved event-free survival and overall survival compared with the effects noted in normal samples or samples with lower miRNA expression levels (39), which suggested its potential application as a biomarker for predicting the outcome in patients with early breast cancer. High miRNA expression levels of miR-100-5p in insulin-like growth factor binding protein 6-knockdown MDA-MB-231 cells were reported to be correlated with the decrease of insulin receptor and cyclin D1 genes which were associated with the insulin-like growth factor signaling pathway and the proliferative and migratory activity during the metastatic cascade in breast cancer (40). Upregulation of miR-146a-5p expression modulated by the methyltransferase 14, N6-adenosine-methyltransferase subunit promoted cell migration and invasion by breast cancer cells (41). Downregulation of miR-130a-3p was previously evaluated in TNBC cells and was compared with the expression noted in normal cells (42), while the present study showed that miR-130a-3p expression was upregulated in TNBC MDA-MB-231 cells compared with the MCF-7 luminal A subtype cells ($ER^+/PR^+/HER2^-$). Overexpression of miR-130a-3p was reported to reduce the proliferation, anchorage-independent growth and migratory activity by

downregulating the Wnt signaling cascade in TNBC cells (42). Therefore, the function of miR-130a-3p warrants further evaluation.

With regard to the downregulated miRNAs, the present study identified certain downregulated miRNAs, including miR-141-3p and miR-205-5p, which have been reported to be associated with pathogenic roles in previous studies. The expression levels of miR-141-3p were downregulated in breast cancer compared with the corresponding levels noted in adjacent non-tumor tissues (43). Overexpression of miR-141-3p has been reported to inhibit cell proliferation, migration and invasion in MCF-7 and MDA-MB-231 cells (43). The expression of miR-205-5p was also reported to be downregulated in breast cancer compared with normal breast tissues. miR-205-5p exhibited tumor suppressive functions by inhibiting tumor growth and metastasis in breast cancer (44). miR-205-5p was expressed at the lowest level in TNBCs compared with other subtypes (44). A limitation of the present study was the limited number of miRNAs detected on the miRNA array, additional miRNAs that are involved in cancer metastasis may not have been detected by the miRNA array used in the present study.

In summary, the present study indicated that miR-222-3p expression was upregulated in the highly metastatic breast cancer MDA-MB-231 cell line, suggesting that this miRNA may be considered as a potential biomarker of metastatic breast cancer in the future. Suppression of miR-222-3p lowered proliferation and the migratory ability of MDA-MB-231 cells, which highlighted the possible roles of miR-222-3p in supporting these processes during cancer progression in high metastatic breast cancer. The present study provided miRNA profiles that require further evaluation for the determination of their functions in MDA-MB-231 cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PP designed and performed the experiments and drafted the manuscript. CA provided suggestions regarding the experimental design and data interpretation, discussed the results and edited the manuscript. SJ contributed to the conception, design of the works, provided advice and suggestions, and edited the manuscript for important intellectual content. PP and CA confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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