Brief Report

MicroRNA analysis of NCI-60 human cancer cells indicates that miR-720 and miR-887 are potential therapeutic biomarkers for breast cancer

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SUMMARY   MicroRNAs (miRNAs) play a vital role in many biological processes, including cell growth, differentiation, apoptosis, development, differentiation, and carcinogenesis. Since miRNAs might play a part in cancer initiation and progression, they comprise an original class of promising diagnostic and prognostic molecular markers. In order to systematically understand the regulation of miRNA expression in cancers, the current study analyzed the miRNA expression profile in NCI-60 human cancer cell lines. Over 300 miRNAs exhibited unique expression profiles in cell lines derived from the same lineage. This study identified 9 lineage-specific miRNA expression patterns. Moreover, results indicated that miR-720 and miR-887 are expressed at relatively high levels in breast cancer cell lines compared to other types of cancer. Ultimately, matching NCI-60 drug response data to miR-720 and miR-887 expression profiles revealed that several FDA-approved drugs were inversely related to miR-720 and miR-887. Furthermore, the anti-cancer effect of perifosine was significantly enhanced by inhibiting miR-720 and decreased by miR-720 precursor treatment in breast cancer cell lines. 5-Fu treatment was enhanced by inhibiting miR-887 and decreased by miR-887 precursor treatment. The current results offer insight into the relationship between miRNA expression and their lineage types, and the approach used here represents a potential cancer therapy with the help of miRNAs.

Keywords   miRNA, NCI-60, lineage-specific, miR-720, miR-887

1. Introduction

MicroRNAs (miRNAs) are small non-coding endogenous RNAs containing 20-25 nucleotides that regulate gene expression at the post-transcriptional level *via* sequence-specific interactions with 3'-untranslatedregions (UTRs) in miRNAs and also *via* inhibition of translation or degradation of miRNAs (*1-3*). miRNAs play a significant role in crucial biological processes, such as cell proliferation, apoptosis, development, differentiation, and metabolism, and miRNAs are especially associated with carcinogenesis (*4-8*). Several studies have indicated that aberrantly expressed miRNAs might serve as oncogenes or tumor suppressor genes in cancers (*9-12*).

NCI-60 from the Developmental Therapeutics Program (DTP) of NCI/NIH, which includes nine types of cancer lines (breast, central nervous system (CNS), colon, leukemia, melanoma, lung, ovarian, prostate, and renal) (*13,14*). Multiple high-throughput screening data is used in the NCI-60 cell line panel, including compound screening data (*15,16*), gene expression data (*17-19*),

data on changes in the DNA copy number (*20*), protein analysis (*21*), DNA methylation (*22*), functional target analysis (*23*), and microRNA expression (*19,24*). This provides many opportunities at the molecular and genetic levels to identify specific pathways and mechanisms associated with cancer (*21,25*).

Because miRNAs are involved in the pathogenesis of cancer, they may be potential molecular biomarkers (*26-28*). miRNAs may help to classify cancers and predict their therapeutic response and also to identify novel targets (*29-31*). In *silico* models have provided a helpful tool for biomedical research (*32,33*). Here, data on miRNA expression by NCI-60 were used to determine the degree of miRNA enrichment in each pedigree, and 9 specific patterns of miRNA expression were identified. Moreover, matching NCI-60 drug response data to miR-720 and miR-887 expression profiles revealed several FDA-approved drugs that were closely inversely correlated with miR-720 and miR-887. Interestingly, the anti-cancer effect of perifosine treatment was improved by inhibiting miR-720 and decreased by miR-

720 precursor treatment in breast cancer cell lines. Inhibition of miR-887 can improve the anti-cancer effect of 5-Fu treatment, while inhibition of miR-887 precursor treatment can reduce the anti-cancer effect of 5-Fu treatment, especially in breast cancer cell lines. The current results provide insight into the relationship between miRNA expression and their lineages, and the approach used here can identify candidates with which to investigate drug resistance and mechanisms of sensitivity in the future.

2. Materials and Methods

2.1. Data acquisition and analysis

The GSE26375 dataset of NCI-60 miRNA expression data was obtained from Gene Expression Omnibus (GEO, *https://www.ncbi.nlm.nih.gov/geo/*). NCI-60 drug response data were obtained from the NCI/NIH DTP program (*www.dtp.nci.nih.gov*). The dataset, published in July 2012, provides the GI50 values describing the sensitivity of NCI-60 DTP human tumor cell lines to 50,839 compounds.

In order to compare the level of miRNA expression in different cancer lineages, the data on NCI-60 miRNA expression were subjected to cell line enrichment analysis (CLEA) (*34*). The priority of cell lines with specific lineages was analyzed on a receiver operating characteristic curve (ROC plot). The area under the ROC curve (AUC) is used to measure the "overexpression" of a given lineage. The *p*-value was used to calculate significance assessed through 1,000 permutations. An AUC value of 50 represents random enrichment. In the analysis of drug response data and miRNA expression data, Pearson's correlation coefficient (PCC) and its *p*-value were calculated.

2.2. The Kaplan-Meier plotter survival analysis

Kaplan Meier plotter (*http://kmplot.com/analysis/*) was used to determine the recurrence-free survival rate (RFs), and the prognostic value of a high level of miRNA expression specifically in Brest cancer samples was also evaluated. The Kaplan–Meier survival curve was plotted, and the log-rank *p* value and hazard ratio (HR) with 95% confidence intervals were calculated and plotted in R using the Bio-conductor package.

2.3. Cell line culture

All cells were purchased from ATCC, and their identity was confirmed and they were tested for contamination prior to shipment. Cells (A549, H460, H322M, MCF-7, T47-D and MDA-MB-231) were grown in RPMI medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/

streptavidin (Gibco; Thermo Fisher Scientific, Inc.), and maintained at 37°C in a humidified atmosphere containing 5% CO_2.

2.4. Quantitative real-time PCR (qPCR) analysis

In accordance with the manufacturer's instructions, RNA was isolated from tissues or cells using the mirVana miRNA Isolation Kit (Ambion; Thermo Fisher Scientific, Inc.). First-strand cDNA was synthesized using a PrimeScript 1st Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Dalian, Liaoning, China). The extraction concentration was determined with a NanoDrop spectrophotometer. The products were kept at –80°C before further experiments. The cDNA was then amplified using the Power SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the appropriate primers and an ABI 7500-fast thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). U6 served as the internal control. Relative expression was measured using the $2^{-\Delta\Delta CT}$ method (*35*).

2.5. MTT assay

Cell proliferation was determined using an MTT assay. 5-Fu and perifosine were purchased from Abmole (Abmole Bioscience Inc.; Houston, TX, USA). The miRNA precursor (has-miR-720 and has-miR-887), precursor-negative control, and miR-720 and miR-887 inhibitors were purchased from Biomics (Biomics Biotech; Jiangsu, Nanjing, China). The cells were seeded on a 96-well plate at a density of 2×10^3 cells per well in triplicate. After culturing for 24 h, the cells transfected with a miRNA precursor or miR-720 and miR-887 inhibitor and then treated with or without perifosine and 5-Fu. Cells were incubated for another 72 h and then their viability was measured using the MTT assay. Twenty μL of MTT (5 mg/mL) was added to the wells, and 4 h later the mixed medium was replaced with 150 μL of dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Subsequently, the 96-well plate was stirred at room temperature for 15 min. Then, the OD value of each well was determined using a fluorescence microplate reader (Sunrise Remote; Tecan Austria GmbH, Grödig, Austria) at a wavelength of 490 nm. All experiments were performed in triplicate.

2.6. Software support

Hierarchical clustering of miRNA expression in NCI-60 cancer cell lines or lineage types was performed using QCanvas for the best presentation (*36*). All images were formatted with Adobe Illustrator CS4 (Adobe Systems, Inc., San Jose, CA, USA). In order to determine significant differences between two groups, a Student's

t-test was performed to calculate the associated *p*-values. Statistical significance between multiple groups was evaluated using one-way analysis of variance (ANOVA) followed by a Newman-Keuls post hoc test using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results and Discussion

3.1. Clustering of NCI-60 cell lines based on miRNA expression

The NCI-60 miRNA expression profile consisted of > 1,000 miRNAs against 59 cancer cell lines. The unsupervised hierarchical clustering of the miRNA expression profile revealed that cell lines from the same lineage were generally located together, and this was especially true for CNS, renal, colon, ovarian, and breast cancer and melanoma (Figure 1A). To identify the expression patterns of lineage-specific miRNA, subsets of miRNAs were selected using CLEA analysis. The enrichment score (AUC value) in CLEA analysis was used to select lineage-specific miRNAs. An AUC value of 85 and a *p*-value of 0.01 were used as cutoff values to ensure that miRNAs had significant over-expression for a particular lineage type while an AUC value of 15 and a *p*-value of 0.01 were used as cutoff values for significant under-expression. As a result, a total of 332 miRNAs were found to satisfy the aforementioned criteria. These selected miRNAs were clustered hierarchically to classify the 9 pedigrees (Figure 1B).

3.2. Breast-specific miRNAs

A total of 13 miRNAs exhibited a breast cancer-
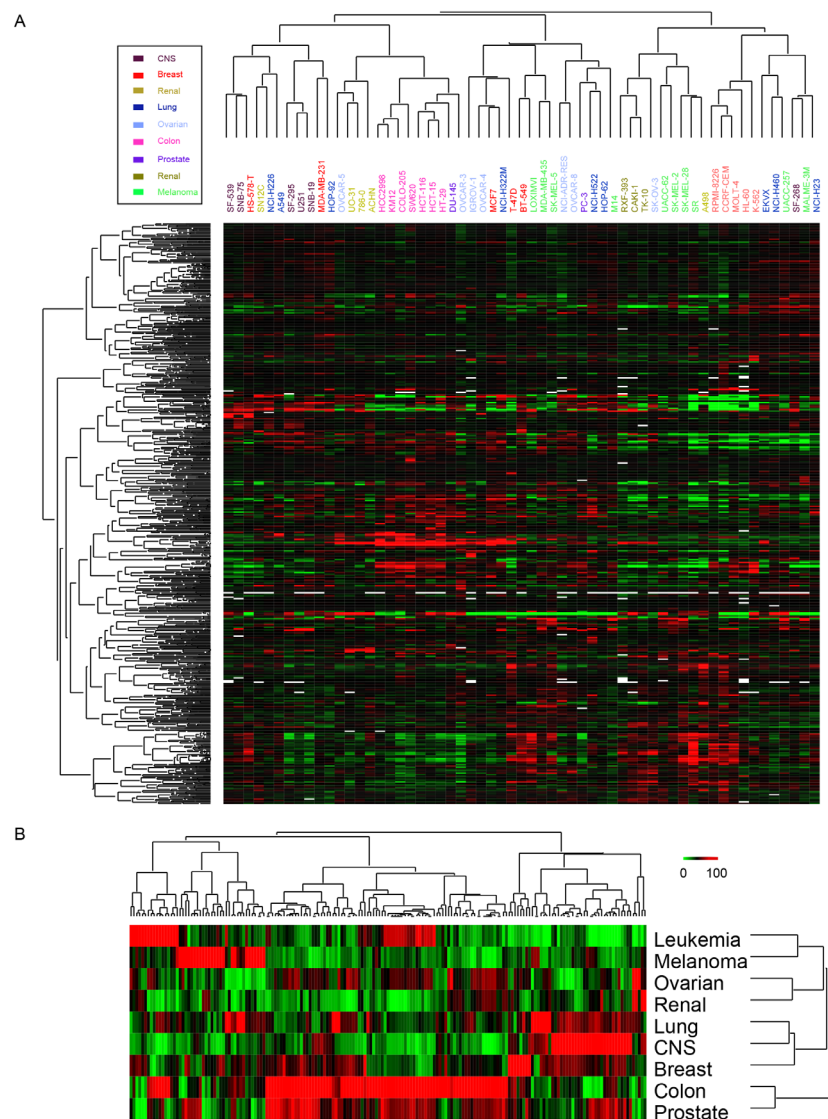
Figure 1. Hierarchical clustering of miRNA expression. (A) Expression profile of total miRNAs across NCI-60 cancer cell lines. **(B)** Expression profile of lineage-specific miRNAs across 9 lineage categories. Red represents over-expression, and green represents a down-expression.
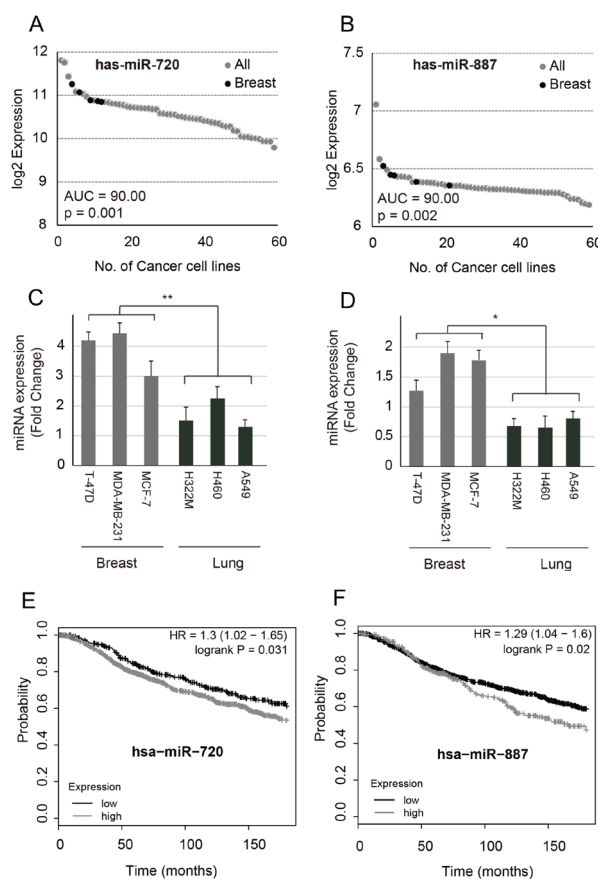
Table 1. List of miRNAs specific to breast cancer

Gene ID	Gene Symbol	AUC	*p*-value
17583	hsa-miR-720	90	0.0013
17873	hsa-miR-887	90	0.0021
27672	hsa-miR-615-3p	91.4815	0.0013
21511	hsa-miRPlus-E1013	90	0.0008
17583	hsa-miR-720	90	0.0013
13148	hsa-miR-195	88.5185	0.0013
10941	hsa-miR-135a	88.5185	0.0016
28520	hsa-miR-454*	87.7778	0.0027
11121	hsa-miR-489	86.6667	0.0027
21704	hsa-miRPlus-E1233	85.9259	0.0025
27550	hsa-miR-572	85.9259	0.0041
17900	hsa-miR-1307	85.5556	0.0023
17943	hsa-miR-760	13.7037	0.0015

Figure 2. miR-720 and miR-887 are expressed at high levels in breast cancer cells. (A) miR-720 and **(B)** miR-887 are expressed at significantly high levels in breast cancer cell lines compared to other NCI-60 cancer cell lines. qPCR analysis of levels of **(C)** miR-720 and **(D)** miR-887 expression in lung cancer cells and breast cancer cells. The prognostic values of **(E)** miR-720 and **(F)** miR-887 in breast cancer. Data are expressed as the mean ± SEM ($n = 3$). $^*p < 0.05$ and $^{**}p < 0.01$ between the compared data.

specific pattern of expression (Table 1). miR-720 and miR-887 were significantly over-expressed in breast cancer cell lines (Figures 2A and 2B). Several studies have found that miR-720 and miR-887 may be tumor suppressors in human cancers, such as renal cell carcinoma (*37*), cervical cancer (*37*), colon cancer (*38*), and breast cancer (*39*). To assess whether or not miR-720 and miR-887 are expressed at high levels in breast cancer, qPCR was used to detect miR-720 and miR-887 expression in 3 breast cancer cell lines and 3 lung cancer cell lines. miR-720 and miR-887 were significantly up-regulated in breast cancer cells (Figures 2C and 2D). Next, the prognostic value of miR-720 and miR-887 was assessed in breast cancer. High levels of miR-720 and miR-887 expression were correlated with a longer relapse-free survival (RFS) in all patients with breast cancer (HR = 1.3, $p = 0.031$ and HR = 1.29, $p = 0.02$) (Figures 2E and 2F).

3.3. miRNAs and drug response

The NCI-60 drug response dataset includes FDA-approved anticancer drugs. The expression profiles of miR-720 and miR-887 were compared to the drug response of the FDA-approved NCI-60 cancer cell line using PCC. The response to 6 drugs (chlorambucil, carmustine, perifosine, doxorubicin, plicamycin, and romidepsin) was inversely related to the expression of miR-720 (Figure 3A). The response to 4 drugs (chlorambucil, doxorubicin, 5-fluorouracil (5-Fu), and mitotane) was inversely related to the expression of miR-887 (Figure 3B). These findings may indicate that cell lines initially express high levels of miR-720 and miR-887 but that further treatment reduces those levels, indicating sensitivity to the drugs used. Perifosine is a third-generation oral alkyl phospholipid with antitumor activity (*40-42*). Perifosine has been found to be an effective and consistent Akt inhibitor in preclinical and clinical studies (*42-45*). In particular, perifosine has been found to be cytotoxic in mouse glioma, medulloblastoma, and neuroblastoma models (*45*). 5-Fu is one of the earliest and still most commonly used

anticancer drugs (*46,47*). The current study verified that the anti-cancer effect of perifosine was significantly enhanced by inhibiting miR-720 (Figure 4A) and that the anti-cancer effect of 5-Fu was significantly enhanced by inhibiting miR-887 in breast cancer cells (Figure 4B). Therefore, miR-720 and miR-887, as suppressors of breast cancer, play an important role in the anti-tumor activity of chemotherapy drugs.

4. Conclusion

The biological characteristics of the NCI-60 cell lines are reflected in their miRNA landscape, as lineage-specific miRNA features are retained across all cell lines. The current study identified a number of miRNAs in each histology that displayed a lineage-specific pattern. This study determined the correlation between miR-720 and miR-887 expression and sensitivity to FDA-approved drugs. This approach can help identify new chemicals and miRNA-based biomarkers for personalized drugs. The current results indicated that miR-720 and miR-887 were over-expressed in breast
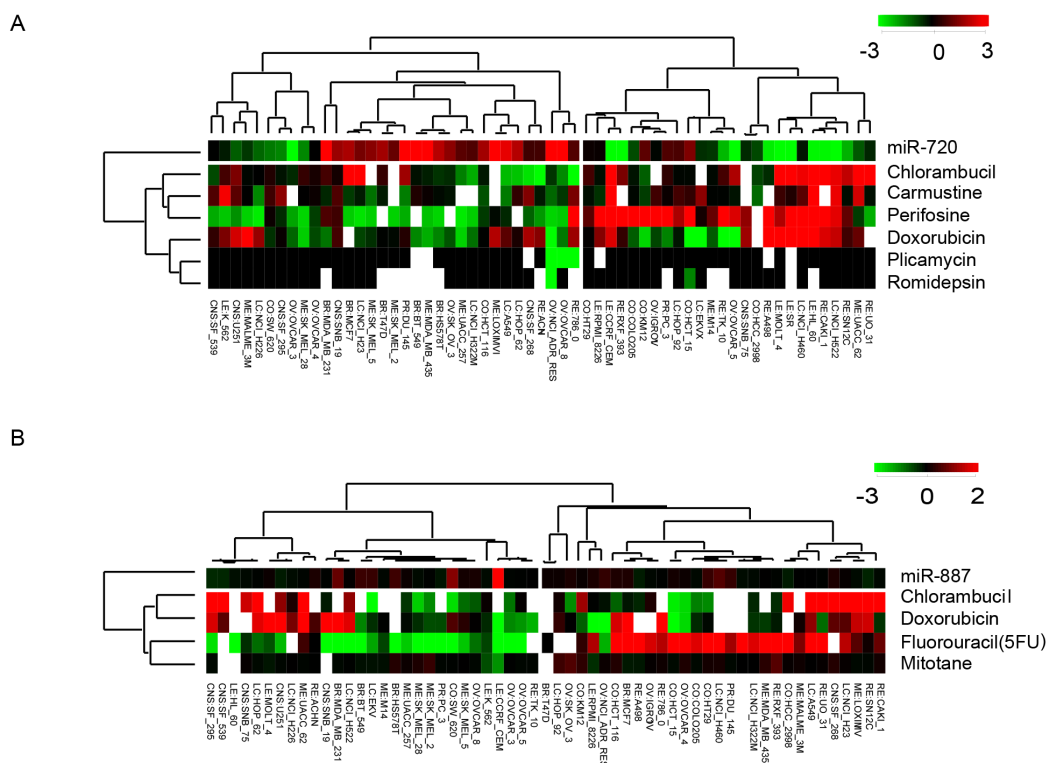
Figure 3. Correlation between miR-720 and miR-887 expression and drug sensitivity. (A) Profile of the drug response of NCI-60, which was negatively correlated with miR-720 expression. **(B)** Profile of the drug response of NCI-60, which was negatively correlated with miR-887 expression. For miRNA expression, red indicates a higher level of expression and green indicates a lower level of expression. For drug response, red indicates a sensitive response, green indicates a resistant response, and white indicates data are not available.
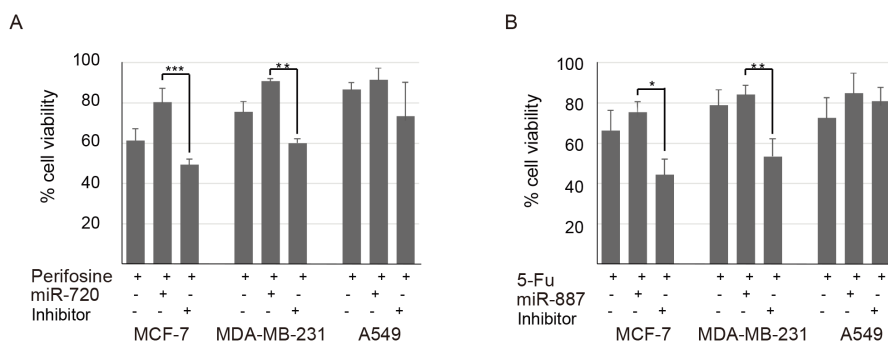
Figure 4. miR-720 and miR-887 as tumor suppressors in breast cancer. Investigation of the clinical potential of perifosine **(A)** and 5-Fu **(B)** combined with miR-720 and miR-887 inhibition for the treatment of breast cancer. Cancer cells were treated with perifosine in the presence of an miR-720 or miR-720 inhibitor and 5-Fu in the presence of miR-887 or miR-887 for 72 hours. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ between the compared groups.

cancers. Testing the combined effect of perifosine with miR-720 inhibition and 5-Fu with miR-887 inhibition revealed the enhancement of anti-cancer action. miR-720 and miR-887 are potent tumor suppressors and may become potential therapeutic tools for patients with breast cancer.

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