Basic Investigations

Aidi Injection (艾迪注射液) Alters the Expression Profiles of MicroRNAs in Human Breast Cancer Cells

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Objective: To investigate the effects of Aidi Injection (艾迪注射液 ADI) on the MicroRNAs (miRNA) expression profiles in human breast cancer cells and explore the potential targets of the cancer treatment.
Methods: MCF-7 breast cancer cells were grown in RPMI 1640 medium supplemented with different concentrations of ADI. The inhibition of cell proliferation was measured by MTT assay. MCF-7 cells were treated by ADI with above 50% inhibiting concentration (IC_{50}) for 48 h. The expression profiles of miRNA in ADI-treated and ADI-untreated MCF-7 cells were detected with miRNA microarray chips and the array data were verified by quantitative RT-PCR. MCF-7 cells were transiently transfected with miRNA mimics by liposome method. Potential mRNA targets were predicted by informatics analysis with TargetScan and PicTar software.
Results: ADI significantly inhibited the proliferation of MCF-7 cells in a dose-dependent manner. The IC_{50} of ADI was 55.71 mg/mL after treatment for 48 h. The 60 mg/mL ADI was used as the therapeutic drug concentration. Microarray analysis identified 45 miRNAs that were up-regulated and 55 miRNAs that were down-regulated in response to ADI treatment. Many ADI-induced miRNAs were related to breast cancers. The microarray data were validated by qRT-PCR. Ectopic expression of 100 nmol/L mir-126 mimics significantly inhibited the proliferation of MCF-7 cells. The 12 potential target genes of mir-126 were predicted by both TargetScan and PicTar software.
Conclusions: The miRNA may serve as therapeutic targets, and the modulation of miRNA expression is an important mechanism of ADI inhibiting breast cancer cell growth.

Keywords: *Aidi Injection; breast cancer cells; miRNA*

Chinese herbal medicine is widely used to treat cancer in China, with a certain effect[1], and it has become one of main ways for cancer comprehensive treatment program. Strengthening the body resistance to eliminate pathogenic factors is a basic principle of Chinese herbal medicine for treatment of cancer.[2,3] Aidi Injection (艾迪注射液 ADI) made by extraction of Ren Shen (Radix Ginsheng), Huang Qi (Radix Astragali), Ci Wu Jia (Radix Acantropanacis Senticosi), Ban Mao (Mylabri) is often used for clinical treatment of cancer.[4,5] It is reported that ADI can inhibit growth of tumour and induce apoptosis, reverse multi-drug resistance, decrease the side-effect of radiotherapy or chemotherapy, increase immune function and improve life quality of the patient.[6-9] The mechanisms of ADI anti-tumour activity have not, however, been completely defined.

MicroRNAs (miRNAs) are a class of non-coding single chain small RNA molecules that function pos-ttranscriptional regulation of gene expression by binding to the 3'UTRs of their target mRNAs.[10,11] In normal tissues, proper miRNA transcription, processing and binding to complementary sequences on the target mRNA result in the repression of target-gene expression through inhibition of protein translation or altering mRNA stability, so as to make cellular growth, proliferation, differentiation and cell death maintain at a normal level.[12] Abnormal miRNA expression is related with various cancers, and these genes are thought to function as both tumour suppressors and oncogenes. Aberrant expression of miRNAs is involved in proliferation, infiltration, apoptosis and signal transduction pathways in breast cancer, and these small regulatory RNAs possibly serve as potential targets of anti-cancer drugs.[13-17] Current report in *Nature* shows that the antiviral mechanism of interferon is through the modulation of cellular miRNAs, suggesting that miRNAs serve as targets for therapeutic intervention.[18] In this study, a microarray chip was used for miRNA expression profiling to test the hypothesis that ADI alters miRNA expression profiles, and it may clarify the antitumor effects of ADI.

METHODS

Drugs and Reagents

ADI (Each milliliter contained 300 mg raw drug. Batch

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No. 20071217) was purchased from Guizhou Ebay Pharmaceutical Co. LTD (China); RPMI 1640 culture medium from Gibco Company; calf serum from PAA Company; MTT from Genview Company; Trypsin from Amresco Company; Trizol reagent and DEPC from BioDev and Invitrogen Companies; TaqMan^{R} Micro-RNA kits from ABI Company; miR-Ribo^{TM} MicroRNA kits, mir-126 mimics, negative control mimics and fluorescence labelled siRNA from Guangzhou Ribobio Co., Ltd (China); HiPerFect transfection reagents from Qiangen company (Germany).

Cell Culture

Human breast cancer MCF-7 cells were purchased from the American Type Culture Collection (ATCC, USA) and cultured in RPMI 1640 medium (Gibco, USA) containing 10% heat-inactivated (56℃, 30 min) fetal calf serum, 2 mmol/L glutamine, penicillin (100 U/ml) and streptomycin (100 μg/mL), which was maintained in an incubator at 37℃ with 5% CO_2 in a humidified atmosphere. The culture medium was replaced each three days and the cells at logarithmic growth phase were used for following experiments

Intervention of ADI

MCF-7 cells were seeded in 96-well culture plates at 5×10^3 cells/well. After overnight incubation, the culture medium was replaced and added with final concentrations of ADI, 20 mg/mL, 40 mg/mL, 60 mg/mL, 80 mg/mL and 100 mg/mL, respectively, and a blank control group and a negative control group were set up with 4 duplicate wells in each group. After culture for 48h, cell proliferation was measured with MTT assay. The experiment was replicated 3 times. The 50% inhibiting concentration (IC_{50}) was calculated according to the effects of ADI inhibiting cell proliferation. MCF-7 cells intervened with ADI of a concentration of over IC_{50} for 48h were used for following Microarray assay.

Detection of Cell Proliferation

Cell viability was measured by the MTT assay (Genview). Cell proliferation was detected by the addition of 20 μl MTT (5 g/L) into each well at 37℃ for 4 h. After the culture medium was removed, DMSO (150 μL) was added and shaken with protecting from light to completely dissolve the formazan crystals. Absorbance (A) was measured at 490 nm with an ELISA plate reader (BioTek, Winooski, VT, USA). For each group, the experiment was repeated thrice with 4 duplicate wells. The percentage of inhibition was calculated as follows: Inhibition rate (IR) = $(1-OD_{Sample}/OD_{Control})\times100\%$. The IC_{50} value was determined by IC_{50} calculator according to the results of the MTT assay.

RNA Extraction and Qualitative Detection

Briefly, the cells were collected and washed with cold PBS buffer. Total RNA was isolated from 5×10^6 cells using Trizol according to the directions (Invitrogen). Absorbance (A) of the total RNA sample was measured by a spectrophotometer respectively at wavelengths of 230, 260, and 280 nm to determine purification and concentration of the total RNA sample. Ratio of 28S to 18S in the total RNA sample was detected by electrophoresis on a 1% agarose gel containing formaldehyde to evaluate its purification and integrity. The RNA sample conforming to quality requirement was used for microarray assay.

Microarray Assay of miRNA Expression Profiles

Microarray assay was carried out by LC Sciences Biotech Co. with a μParaflo™ microfluidic chip. 5 μg of total RNA sample was fractionated using a YM-100 Microcon centrifugal filter (Millipore), attaining the isolated small RNAs (<300 nt), which were 3'-extended with a poly (A) polymerase. An oligonucleotide tag was then ligated to the poly A. Hybridization was performed overnight on a μParaflo™ microfluidic chip using a micro-circulation pump (Atactic Technologies). On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to target microRNA from the miRBase Sequence database version 10.0 (Sanger Institute, Cambridge, U.K.; http://microrna.sanger.ac.uk/sequences). The detection probes were made by in situ synthesis using PGR (photogenerated reagent). Each probe sequence was repeated five times on the same array chip. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization was carried out with 100 μL 6 × SSPE buffer (0.90 mol/L NaCl, 60 mmol/L Na_2HPO_4, 6 mmol/L EDTA, pH 6.8) containing 25% formamide at 34℃. The specific fluorescence labelling with tag-specific Cy3 and Cy5 dyes was used for hybridization detection. Hybridization images were collected using a laser scanner (GenePix 4000B, Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics).

Processing and Statistical Analysis of Microarray Data

The data were analyzed after subtracting the background, the mean of duplicate points and the standard deviation were calculated and normalizing the signals using a LOWESS filters (Locally-weighted Regression). For bicolour labelling experiments, the ratio of the two detected signals (log_2 transformed) and *P*-values of the *t*-test were calculated. A *P*-value less than 0.05 was considered as statistically significant. Log_2 with positive number expressed as up-regulation and log_2 with negative number as down-regulation.

qRT-PCR for miRNA Analysis

Real-time PCR-based quantification of miRNA was performed using miRNA analysis kits specific for each individual miRNA (Applied Biosystems) and Bulge-Loop^{TM} miRNA qPCR Primer Set (Guangzhou Ribobio

Co., Ltd, China) according to the instructions. Quantitative miRNA expression data were detected and analysed using a LightCycler 1.5 fluorescent quantification PCR instrument (Roche Applied Science).

Transient miRNA Transfection

MCF-7 cells at logarithmic growth phase were inoculated at 6×10^3 cells per well in 96-well plates with four duplicate wells in each group. For each transfection, MCF-7 cells were transfected with 100 nmol/L mir-126 mimics, negative control mimics and fluorescently double labelled siRNA (Guangzhou RiboBio Co.,Ltd, China) with HiPerFect transfection reagent (Qiangen) following the instructions. The fluorescently labelled molecules were used for monitoring transfection of MCF-7 cells. After transfection for 48 h, cell viability was measured by the MTT assay. The transfection experiment was repeated thrice.

Prediction of miRNA Target Genes

The target genes of differential expression miRNAs were predicted by TargetScan and PicTar software following the instructions in TargetScan (http://www.targetscan.org) and PicTar (http://pictar.bio.nyu.edu).[19, 20]

Statistical Analysis

All the results were expressed as mean ± standard deviation (SD). Statistical analysis was done with Student's *t*-test for comparison of two groups. In all the test, differences with $P<0.05$ were considered statistically significant.

RESULTS

ADI Treatment Inhibited the Proliferation of MCF-7 cells

The MTT assay was used to determine the effects of ADI on MCF-7 human breast cancer cell growth. Figure 1 illustrates the effects of 20-100 mg/mL ADI on the growth of MCF-7 cells after 48 h of incubation. After 48 h of incubation, ADI inhibited the proliferation of MCF-7 cells in a dose-dependent manner. While ADI concentrations of 20-100 mg/mL, the cell numbers were reduced from 3 (±3) % to 93 (±7) %. The 50% inhibiting concentration (IC_{50}) of ADI was 55.71 mg/mL after incubation for 48 h. The 60 mg/mL dose of ADI was used as the therapeutic concentration.

ADI Treatment altered miRNA Expression Profiles in MCF-7 Cells

To assess miRNAs response to ADI, microarray analysis of miRNA expression was conducted with miRNA-enriched total RNAs extracted from human breast adenocarcinoma MCF-7 cells treated with 60 mg/mL ADI for 48 h. Compared with the control group, there were 100 differentially expressed miRNAs in the ADI treatment group. Among them, 45 miRNAs were up-regulated and 55 miRNAs were down-regulated significantly ($P<0.05$), miRNAs down-regulated being more than up-regulated for ADI treatment.

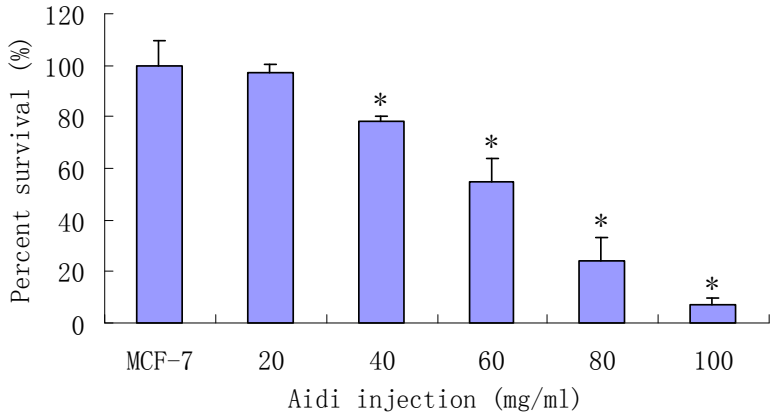

Figure 1. ADI significantly inhibited the proliferation of MCF-7 cells. The results are expressed as cell percent survival relative to the control (mean±SD of three independent experiments) (*$P<0.01$)

Verification of Differentially Expressed miRNAs by qRT-PCR

To validate the microarray data, 5 miRNAs, mir-18a, mir-125b, mir-126, mir-181d and mir-195, were selected at random and their expression levels were assayed by qRT-PCR, and U6 was used for normalization. The results from the microarray and qRT-PCR were compared. The results showed that among the miRNAs selected for comparison, 4 miRNAs (mir-125b, mir-126, mir-181d and mir-195) were up-regulated whereas 1 miRNAs (mir-18a) was down-regulated. The expression data obtained by qRT-PCR analysis are comparable to the microarray analysis data, although mir-195 was up-regulated to a lesser degree in the qRT-PCR analyses (Figure 2).

Mir-126 Repressed Proliferation of MCF-7 Cells

In order to determine the effect of mir-126 on MCF-7 cell proliferation activity, MCF-7 cells were transiently

transfected with 100 nmol/L mir-126 and negative control miRNA mimics. 48 h later, MCF-7 cell activities were detected with MTT and the effect of the mir-126 was compared with that of the negative control mimics. It was found that mir-126 molecules could significantly inhibit the growth of the MCF-7 cell proliferation activity , with a cell survival rate of about 86% (Figure 3).

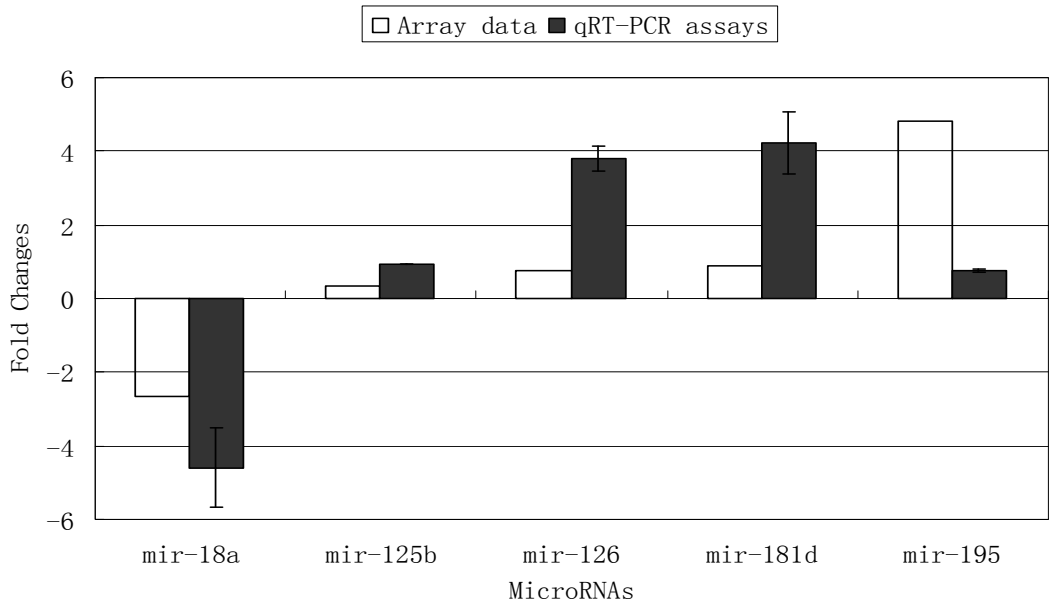

Figure 2. Comparison of results of microarray and qRT-PCR. The fold changes refer to the expression fold changes of the selected miRNAs in ADI-treated cells compared with untreated control cells. The results are expressed as mean ± SD of three independent experiments (P<0.01).

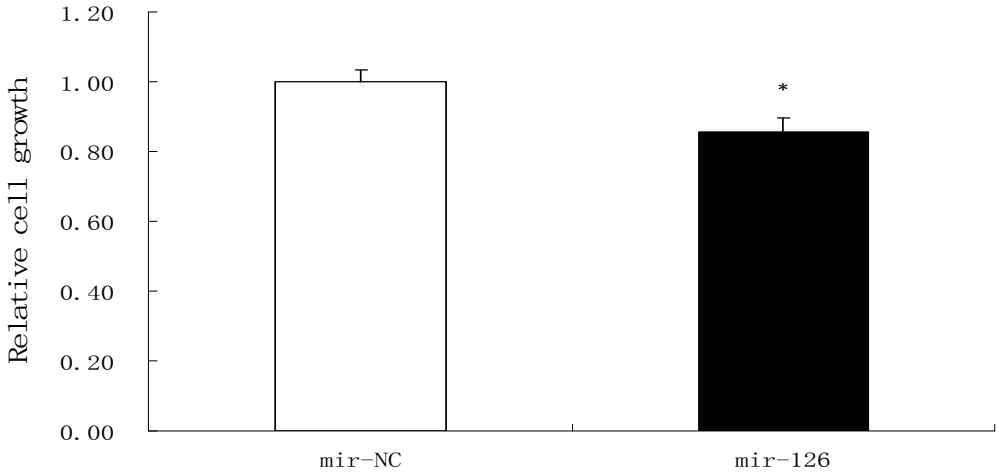

Figure 3. Mir-126 inhibited the proliferation of MCF-7 cells. The breast caner cell line MCF-7 was transfected with mir-126 mimics and negative control mimics (100 nmol/L). mir-126 could inhibit the cell growth (*P<0.01). Take negative control as 100%.

Predictions for the Target Genes of mir-126

Mir-126 is down-regulated in breast cancer cell lines and it can repress cancer cell proliferation.[21] In both microarray and qRT-PCR analyses, mir-126 was significantly up-regulated in MCF-7 cells after treatment with 60 mg/mL ADI for 48 h. This suggests that mir-126 may play an important function in MCF-7 response to ADI. Thus, the two computational programs (TargetScan and PicTar) were employed to predict the targets of mir-126. In order to reduce sham positive rate, the genes commonly occurred in the two software were regarded as possible target genes. The 12 target genes predicted by the both programs are more likely to be the real targets of the miRNA, which were shown in Table 1.

Table 1. Potential target genes of mir-126 predicted by PicTar and TargetScan.

Target gene	RefSeq ID	Target gene name	Target gene	RefSeq ID	Target gene name
IRS1	NM_005544	Insulin receptor substrate 1	FBXO33	NM_203301	F-box only protein 33
PLK2	NM_006622	polo-like kinase 2	GOLPH3	NM_022130	Golgi phosphoprotein 3
PHF15	NM_015288	PHD finger protein 15	SPRED1	NM_152594	Sprouty-related, EVH1 domain-containing protein 1
PTPN9	NM_002833	Tyrosine-protein phosphatase non-receptor type 9	CRK	NM_005206	Proto-oncogene C-crk (P38)
ITGA6	NM_000210	Integrin alpha-6 precursor (VLA-6)	TOM1	NM_005488	Target of Myb protein 1
RGS3	NM_017790	Regulator of G-protein signaling 3	SLC7A5	NM_003486	Large neutral amino acids transporter small subunit 1

DISCUSSION

Cancer is a class of diseases, characterized by cell uncontrolled growth, infiltration, and sometimes metastasis, which are caused by inactivation of tumour suppressor genes and abnormal expression of oncogenes. Previously, it was held that protein-coding oncogenes and tumour suppressor genes were causes of tumour generation. With the discovery of non-protein-coding RNA, the traditional concept is being challenged. It is increasingly recognized that miRNAs, members of non-protein-coding RNAs, play important roles in formation of cancer .

miRNAs are important regulators of gene expression, and they show effects of tumour suppressor genes and abnormal expression of oncogenes through regulating the expression of downstream target genes, including transcription factors, oncogenes, and tumour suppressor genes.[22,23] Thus, the miRNA with oncogenic or tumour suppressive action may be used as a therapeutic target to control cancers.[17] Previous studies on molecular pharmacology of TCM for treatment of breast cancer have much focused on the alterations of expression of tumour-related protein-coding genes.[24-26] However, very few studies were involved in non-coding miRNA with function of regulating gene expressions. Chinese herbal medicine has definite therapeutic effects on cancer, so, the explanation of the anti-cancer effect by regulating miRNA and the mediating molecular pathway will provide a new research thinking and theoretical basis for cancer treatment with Chinese herbal medicine.

ADI is an injection of Chinese herbal medicine with multi-components, which is prepared by the extract of Ren Shen (Radix Ginsheng), Huang Qi (Radix Astragali), Ci Wu Jia (Radix Acantropanacis Senticosi) and Ban Mao (Mylabri), with unclear mechanisms. Since it is difficult to evaluate the effects of Chinese herbal medicine with multi-components, in the study the anti-cancer mechanism of ADI was investigated through a holistic evaluation of miRNA expressions, so as to explore potential treated targets of ADI.[9]

The results in the study indicated that the expressions of 100 miRNAs in MCF-7 cells were regulated by ADI. Through literature search, it was found that the ADI-regulated miRNAs in this study had been reported by the previous studies.[13-16, 27-31] Many breast cancer-related miRNAs were significantly affected by ADI exposure. For example, the expressions of mir-27a, mir-181b, mir-191, and mir-210 were up-regulated in human breast cancer, [27,28] but they were significantly down-regulated by ADI. mir-155 with an oncogenic role was up-regulated in human breast cancer,[27,29] but it was significantly down-regulated by ADI in this study. The expressions of miRNA let-7, mir-125b, mir-126 and mir-7 were down-regulated in human breast cancer with cancer suppressor gene action.[27, 30] let-7 miRNAs were down-regulated in the breast cancer patient with either lymph node metastasis or higher proliferation index.[27] It has been proved that the RAS, HMGA2 and MYC oncogenes are 3 target genes of the let-7 miRNAs,[32-34] which influence proliferation of cancer cells through regulating target genes. mir-126 is identified as metastasis suppressor microRNA in human breast cancer[35] and it regulates expression of target gene IRS-1 to inhibit proliferation of cancer cells.[21] mir-7 is a suppressor microRNA in human breast cancer[30] and it targets Pak1[30] and EGFR[36] to produce cancer-inhibiting action. The potential tumour suppressor mir-125b targets oncogenes ERBB2 and ERBB3 in breast cancer cells.[37] In this study, expressions of let-7RMA, mir-126, mir-7 and mir-125b, etc. were significantly up-regulated by ADI. Thus, ADI-induced miRNAs display an anti-tumour activity for breast cancer.

It may be expected that targets of miRNAs up-regulated by ADI possibly are oncogenes or genes encoding proteins with potential oncogenic functions. It was reported that mir-126 was down-regulated in breast cancer cell lines.[21] mir-126 was up-regulated by ADI and it had potential roles as a tumour-suppressor gene in tumour genesis. It is showed that cancer-associated genes were potentially regulated by mir-126. Therefore, mir-126 was selected for preliminary bio-informatics analysis and it was found that its targets included the IRS1, SLC7A5 and CRK genes. However, the regulated precise target genes remain to be further confirmed.

In brief, miRNA is a key component in genes regulating networks and it has functions of tumour suppressor genes or oncogenes in breast cancer genesis. Most related researches have showed that miRNAs are associated with breast cancer and can serve as the potential targets for cancer treatment. In this study, it was found that ADI altered the expression profiles of miRNAs in human breast cancer cells when ADI treatment effectively inhibited the proliferation of MCF-7 cells. Informatics analysis showed that cancer-associated genes were potentially regulated by mir-126. This suggests that regulation of ADI on miRNAs and the molecular pathway possibly is one of main mechanisms for treatment of breast cancer.

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