Identification of targets of miRNA-221 and miRNA-222 in fulvestrant-resistant breast cancer

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Abstract. The present study aimed to identify the differentially expressed genes (DEGs) regulated by microRNA (miRNA)-221 and miRNA-222 that are associated with the resistance of breast cancer to fulvestrant. The GSE19777 transcription profile was downloaded from the Gene Expression Omnibus database, and includes data from three samples of antisense miRNA-221-transfected fulvestrant-resistant MCF7-FR breast cancer cells, three samples of antisense miRNA-222-transfected fulvestrant-resistant MCF7-FR cells and three samples of control inhibitor (green fluorescent protein)-treated fulvestrant-resistant MCF7-FR cells. The linear models for microarray data package in R/Bioconductor was employed to screen for DEGs in the miRNA-transfected cells, and the pheatmap package in R was used to perform two-way clustering. Pathway enrichment was conducted using the Gene Set Enrichment Analysis tool. Furthermore, a miRNA-messenger (m) RNA regulatory network depicting interactions between miRNA-targeted upregulated DEGs was constructed and visualized using Cytoscape. In total, 492 and 404 DEGs were identified for the antisense miRNA-221-transfected MCF7-FR cells and the antisense miRNA-222-transfected MCF7-FR cells, respectively. Genes of the pentose phosphate pathway (PPP) were significantly enriched in the antisense miRNA-221-transfected MCF7-FR cells. In addition, components of the Wnt signaling pathway and cell adhesion molecules (CAMs) were significantly enriched in the antisense miRNA-222-transfected MCF7-FR cells. In the miRNA-mRNA regulatory network, miRNA-222 was demonstrated to target protocadherin 10 (*PCDH10*). The results of the present study suggested that the PPP and Wnt signaling pathways, as well as CAMs and *PCDH10,* may be associated with the resistance of breast cancer to fulvestrant.

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Introduction

Breast cancer, which accounts for ~23% of all newly diagnosed cases of cancer and was responsible for 14% (458,400) of all mortalities due to cancer in 2008, is the leading cause of cancer-associated mortality among females (1). A previous study reported that the estrogen receptor (ER), which is expressed in ~75% of breast tumors, is considered the main target for the treatment of breast cancer, and women with breast tumors typically receive endocrine therapy (2).

Fulvestrant, which is a pure, steroidal antiestrogen, has been reported to completely suppress ERα activity by inactivating ERα-mediated genomic and non-genomic signaling; it is considered a promising drug for the treatment of breast cancer in postmenopausal women (3). However, ER-targeted therapies fail in ≤50% of patients with breast tumors due to the occurrence of *de novo* or acquired resistance (2,4). It has been reported that microRNAs (miRNAs) have a pivotal role in breast cancer, and the overexpression of miRNA-221/222 has been suggested to be associated with the emergence of fulvestrant resistance in breast cancer (5).

In 2011, Rao *et al* (6) used a microarray expression profile to identify differentially expressed genes (DEGs) between antisense miRNA-221-transfected or miRNA-222-transfected MCF7-FR cells and negative control-transfected MCF7-FR

Table I. Top ten upregulated and downregulated DEGs in the antisense miRNA-221-transfected and antisense miRNA-222-transfected MCF7-FR cells, as compared with negative control-transfected MCF7-FR cells.

	Antisense miRNA-221 vs. control			Antisense miRNA-222 vs. control		
DEGs	Gene	$\|\log_2 FC\|$	adj.P.Val	Gene	$\|\log_2 FC\|$	adj.P.Val
Downregulated	*LHX8*	-4.17614	0.000175	*PTH*	-3.49280	0.000184
	PSMB8	-4.06074	0.000204	*PWAR5*	-3.27792	0.009442
	FLG2	-3.99026	0.001523	*IYD*	-3.20234	0.001261
	TRPC5	-3.83132	0.002291	*ICAM5*	-2.87824	0.017322
	OLFM4	-3.51933	0.000522	*DAOA-AS1*	-2.83576	0.044589
	KERA	-3.37161	0.006165	*STARD13-AS*	-2.79578	0.005935
	GIMAP2	-3.19819	0.008755	*WDR86-AS1*	-2.75129	0.021120
	CYP4F30P	-2.92305	0.042475	*IZUMO2*	-2.68134	0.028787
	LOC100505635	-2.89457	0.041871	*RAG2*	-2.67795	0.006909
	SLC15A3	-2.88269	0.000007	*C1orf192*	-2.66616	0.022020
Upregulated	*PRPS1L1*	3.81365	0.002733	*OR2L13*	3.55457	0.007431
	ARHGAP36	3.33814	0.000913	*PRPS1L1*	3.54601	0.002105
	CXorf58	3.28644	0.000074	*SH3RF3-AS1*	3.34868	0.000066
	LINC00567	3.24582	0.000653	*CXorf58*	3.15695	0.028128
	DYDC1	3.18358	0.026382	*LOC100505676*	3.11364	0.001263
	OR2L13	2.88576	0.003734	*LINC00950*	2.92266	0.001549
	MLIP	2.83880	0.036347	*NXPH1*	2.90566	0.000380
	KLKB1	2.79668	0.015902	*MSTN*	2.89563	0.000007
	LINC00950	2.75805	0.001693	*DZIP1*	2.86233	0.007720
	OR10A5	2.74849	0.011940	*CPEB2-AS1*	2.85775	0.015210

FC, fold change; adj.P.Val, adjusted P-value; miRNA, microRNA; DEGs, differentially expressed genes.

cells, according to the cut-off criteria of P<0.05 and $|\log_2$ fold change (FC)$|$ >1.2. It was demonstrated that activation of β-catenin by miRNA-221/222 led to estrogen-independent growth and fulvestrant resistance, as well as to repression of transforming growth factor-β-mediated growth inhibition (6). However, another study reported different mechanisms for the occurrence of fulvestrant resistance in breast cancer (7). Tangkeangsirisin and Serrero (8) demonstrated that progranulin induced human breast cancer resistance to fulvestrant by inhibiting the apoptosis of breast cancer cells. In addition, the broad-spectrum metalloproteinase inhibitor BB-94 has been demonstrated to inhibit the growth of fulvestrant-resistant breast cancer cell lines, as well as the activation of human epidermal growth factor receptor 3 and extracellular signal-regulated kinase in these cells (9). Therefore, it is important to further screen for biomarkers associated with fulvestrant-resistance in breast cancer.

Using the same microarray data as Rao *et al* (6), the present study aimed to further screen for DEGs in antisense miRNA-221-transfected and antisense miRNA-222-transfected MCF7-FR cells. The linear models for microarray data (limma) package, based on a wide threshold range (P<0.05 and $|\log_2 FC| >1$), was used to identify DEGs associated with fulvestrant-resistant breast cancer. In addition, a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed, and the targets of miRNA-221/222 were predicted using miRanda and TargetScan. A previous study suggested that analyses based on different statistical tests may produce different outcomes (10). Therefore, the present study may obtain a number of results different from the data obtained in the initial study by Rao *et al* (6).

Materials and methods

Microarray data. The GSE19777 transcription profile used by Rao *et al* (6) was downloaded from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/). The profile was based on the GPL570 dataset, which was obtained using the [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Inc., Santa Clara, CA, USA). In total, nine samples were included in the dataset, including three samples of antisense miRNA-221-transfected fulvestrant-resistant MCF7-FR breast cancer cells, three samples of antisense miRNA-222-transfected fulvestrant-resistant MCF7-FR cells and three samples of control inhibitor (green fluorescent protein)-treated fulvestrant-resistant MCF7-FR cells (negative control). In addition, the probe annotation information mapping the probes of genes was downloaded from Bioconductor (http://www.bioconductor.org/).

Dataset preprocessing and DEG analysis. The R package from Affymetrix, Inc., was used to normalize the raw CEL data from the DNA microarrays (11). The downloaded expression profile was mapped to the corresponding

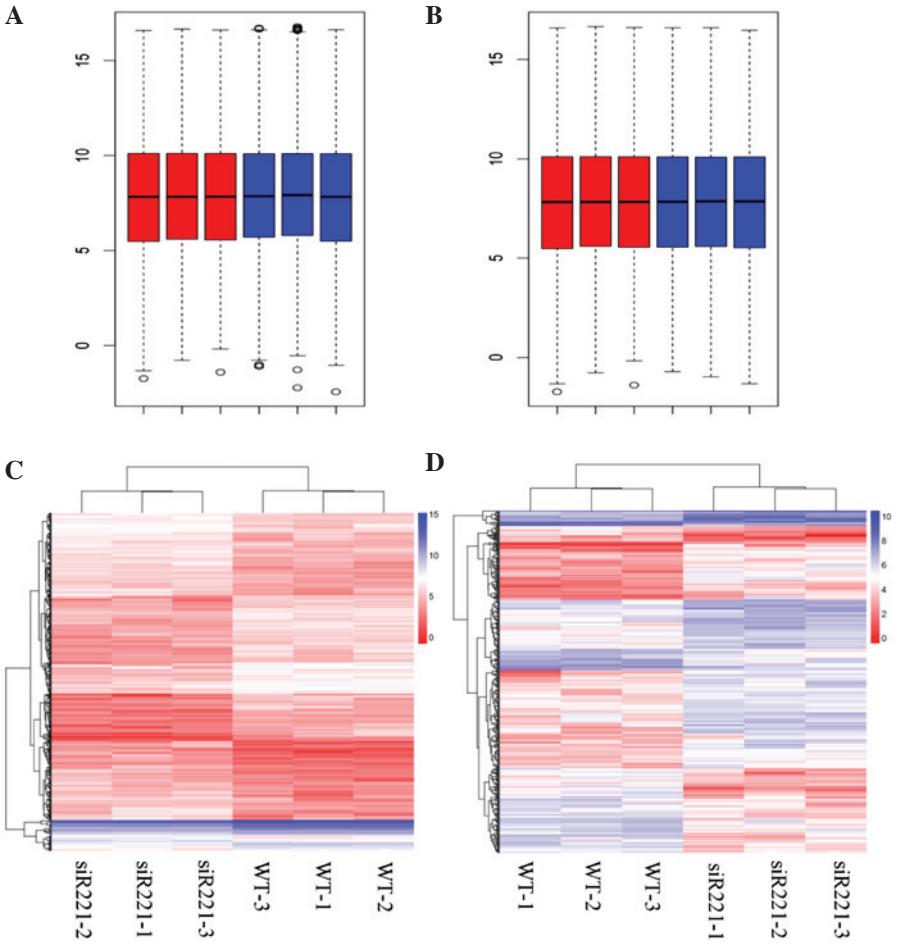

Figure 1. Box plots for the normalized gene expression data and the hierarchical clusters of DEGs. (A) Box plots for the normalized gene expression data of the antisense miRNA-221-transfected MCF7-FR breast cancer cells (red box plots) and the negative control-transfected MCF7-FR cells (blue box plots). (B) Box plots for the normalized gene expression data of the antisense miRNA-222-transfected MCF7-FR cells (red box plots) and the negative control-transfected MCF7-FR cells (blue box plots). (C) Hierarchical cluster analysis of DEGs in the antisense miRNA-221-transfected MCF7-FR cells, as compared with the negative control-transfected MCF7-FR cells. (D) Hierarchical cluster analysis of DEGs in the antisense miRNA-222-transfected MCF7-FR cells, as compared with the negative control-transfected MCF7-FR cells. The horizontal axis represents the samples. The vertical axis represents the DEGs. The color key indicates the expression value of the DEG. siR221, antisense miRNA-221-transfected MCF7-FR cells; DEGs, differentially expressed genes; miRNA, microRNA.

gene symbols. Average expression values were used for the genes with multiple probes. Subsequently, the limma package in R/Bioconductor (https://bioconductor.org/packages/release/bioc/html/limma.html) was used to screen for DEGs in the antisense miRNA-221-transfected and miRNA-222-transfected MCF7-FR cells, as compared with the negative control. The cut-off criteria for the DEGs were $P<0.05$ and $|\log_2 FC| >1$. The top ten upregulated and downregulated genes in the antisense miRNA-221-transfected and antisense miRNA-222-transfected MCF7-FR cells are indicated in Table I. Next, the pheatmap package (https://cran.r-project.org/web/packages/pheatmap/index.html) in R was used to perform two-way clustering (12), based on the Euclidean distance (13).

Gene set enrichment analysis (GSEA). GSEA, which is a computational method that determines whether an *a priori* defined set of genes exhibits statistically significant and concordant differences between two biological states (14), was used to conduct the pathway enrichment analysis based on the expression levels of DEGs in the antisense miRNA-221-transfected and miRNA-222-transfected MCF7-FR cells. A gene count between 15 and 500 and $P<0.01$ were set as the criteria to filter the pre-defined gene sets. In addition, the distant regulatory elements of co-regulated genes tool (http://dire.dcode.org), which enables the prediction of distant regulatory elements in higher eukaryotic genomes (15), was applied to screen for transcription factors associated with the DEGs in the enriched pathways.

miRNA-messenger (m) RNA regulatory network construction. Prediction of the targets of miRNA-221 and miRNA-222 was performed using the miRanda algorithm (http://microrna.sanger.ac.uk/targets/v5/) and TargetScan 4.2 (http://www.targetscan.org/). Subsequently, the miRNA-mRNA regulatory network, depicting interactions between the miRNAs and target DEGs (upregulated DEGs only) was constructed and visualized using Cytoscape (16).

Results

Preprocessing and DEG analysis. The box plots of the expression values for all genes in every sample following normalization are represented in Fig. 1A and B. In total,

Table II. Enriched pathways for DEGs in the miRNA-221-transfected and miRNA-222-transfected MCF7-FR cells, as compared with the negative control-transfected MCF7-FR cells.

Groups	Pathway	Counts	P-value	Top 10 DEGs
Antisense miRNA-221-transfected MCF7-FR cells	PPP	26	0	*RPE, RPIA, PGM2, PGLS, PRPS2, FBP2, PFKM, PFKL, TALDO1, TKT*
	Histidine metabolism	27	0	*CNDP1, MAOB, MAOA, ALDH1B1, ALDH2, METTL6, WBSCR22, HAL, HNMT*
	Olfactory transduction	114	0	*CALM2, CALM1, OR11H4, OR52W1, OR5AU1, ADRBK2, OR2M2, OR2M7, OR2T33, OR4F5*
Antisense miRNA-222-transfected MCF7-FR cells	PPP	26	0	*RPE, RPIA, PGM2, PGLS, PRPS2, FBP2, PFKM, PFKL, TALDO1, TKT*
	Taste transduction	44	0	*TAS2R60, GRM4, PLCB2, ADCY8, ADCY6, TAS2R42, TAS1R2, TAS1R1, TRPM5 ACCN1*
	Propanoate metabolism	31	0	*ACSS2 ALDH1B1, ABAT, LOC283398, ALDH2, ACADM, ACAT2, ACAT1, LDHC, MCEE*
	Wnt signaling pathway	144	0	*JUN, LRP5, LRP6, PPP3R2, SFRP2, SFRP1, PPP3CC, VANGL1, PPP3R1, FZD1*
	Arrhythmogenic right ventricular cardiomyopathy	73	0	*CACNA2D1, CACNB1, LOC100418883, CACNB2, CACNB3, CACNB4, CACNG1, ITGA9, CACNG8, RYR2*
	Axon guidance	127	0	*UNC5B, PLXNB2, PPP3R2, PPP3CC, PPP3R1, PAK4, NGEF, SEMA4C, SEMA4A, PLXNC1*
	Prion diseases	35	0	*NCAM2, EGR1, NCAM1, ELK1, NOTCH1, PRKX, C6, CCL5, C5, IL1B*
	Cell adhesion molecules	125	0	*CDH5, JAM3, CDH3, NLGN3, CDH4, CD80, NLGN1, CD86, CD28, CD274*
	Neuroactive ligand receptor interaction	252	0	*PTGFR, PTGER2, PTGER1, PTGER4, PTGER3, CALCRL, TACR3, PTGIR, ADRB3, ADRB2*
	Olfactory transduction	114	0	*CALM2, CALM1, OR11H4, OR52W1, OR5AU1, ADRBK2, OR2M2, OR2M7, OR2T33, OR4F5*

DEGs, differentially expressed genes; miRNA, microRNA; PPP, pentose phosphate pathway.

Table III. Counts of transcription factors for the differentially expressed genes in the enriched pathways.

Group	KEGG pathway	Counts
Antisense miRNA-221-transfected MCF7-FR cells	PPP	87
	Histidine metabolism	80
	Olfactory transduction	95
Antisense miRNA-222-transfected MCF7-FR cells	PPP	87
	Taste transduction	76
	Propanoate metabolism	76
	Wnt signaling pathway	94
	Arrhythmogenic right ventricular cardiomyopathy	116
	Axon guidance	104
	Prion diseases	79
	Cell adhesion molecules	123
	Neuroactive ligand receptor interaction	113
	Olfactory transduction	95

KEGG, Kyoto Encyclopedia of Genes and Genomes; miRNA, microRNA; PPP, pentose phosphate pathway.

492 DEGs, including 247 upregulated [such as phosphoribosyl pyrophosphate synthetase 1-like 1 (*PRPS1L1*) and secreted frizzled-related protein 5 (*SFRP5*)] and 245 downregulated (such as LIM homeobox 8 and proteasome subunit beta 8) DEGs, were identified in the antisense miRNA-221-transfected MCF7-FR cells compared with the negative control, while 404 DEGs, including 255 upregulated [such as *PRPS1L1* and claudin 8 (*CLDN8*)] and 149 downregulated (such as parathyroid hormone and Prader Willi/Angelman region RNA 5) DEGs, were identified in the antisense miRNA-222-transfected MCF7-FR cells compared with the negative control. The two-way hierarchical cluster analyses of the DEGs in the miRNA-221- and miRNA-222-transfected cells are represented in Fig. 1C and D.

GSEA. Three pathways were significantly enriched in the antisense miRNA-221-transfected MCF7-FR cells compared with the negative control, while ten pathways were significantly enriched in the miRNA-221-transfected MCF7-FR cells compared with the negative control (Table II). In addition, two pathways, including the pentose phosphate pathway (PPP) and olfactory transduction, were enriched in both the antisense miRNA-221-transfected and miRNA-222-transfected MCF7-FR cells, as compared with the negative control (Table II). Notably, the DEGs *SFRP5* and *CLDN8* were significantly enriched in the Wnt signaling pathway and the cell adhesion molecules (CAMs) pathway, respectively (Table II).

Screening for transcription factors associated with the genes in the enriched pathways identified 123 transcription factors associated with the genes in the CAMs pathway. Furthermore, 94 transcription factors were associated with the genes enriched in the Wnt signaling pathway, and 87 transcription factors were associated with the genes enriched in the PPP (Table III).

Regulatory network analysis. According to the TargetScan and miRanda databases, 530 genes were targets of miRNA-221 and 488 genes were targets of miRNA-221. Of the 530 target genes of miRNA-221, six were DEGs, including four upregulated genes [recombination activating gene 1, leucine-rich repeats and calponin homology domain containing 2, methionine sulfoxide reductase B3 (*MSRB3*) and neurexophilin 1 (*NXPH1*)], in the antisense miRNA-221-transfected MCF7-FR cells. Of the 488 target genes of miRNA-222, ten

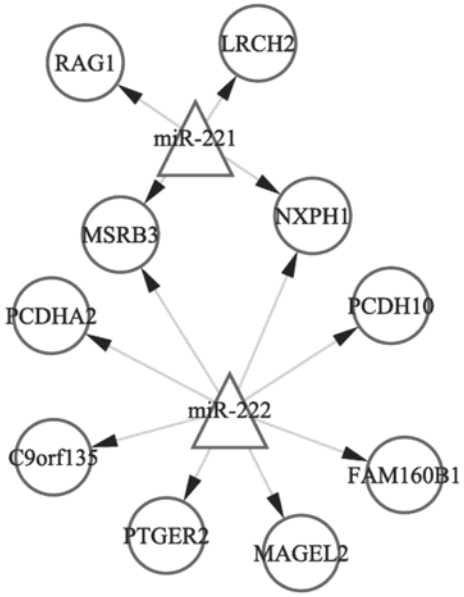

Figure 2. miRNA (miRNA-221 and miRNA-222)-target regulatory network. Triangles represent the miRNAs; circles represent the differentially expressed genes in miRNA-221- and miRNA-222-transfected MCF7-FR breast cancer cells. miRNA, microRNA; RAG1, recombination-activating gene 1; LRCH2, leucine-rich repeats and calponin homology domain containing 2; MSRB3, methionine sulfoxide reductase B3; NXPH1, neurexophilin 1; PCDHA2, protocadherin α-2; PCDH10, protocadherin-10; C9orf135, chromosome 9 open reading frame 135; PTGER2, prostaglandin E receptor 2; MAGEL2, MAGE family member L2; FAM160B1, family with sequence similarity 160 member B1.

were DEGs, including eight upregulated genes (*MSRB3*, *NXPH1*, protocadherin (*PCDH*) *A2*, *PCDH10*, chromosome 9 open reading frame 135, prostaglandin E receptor 2, MAGE-like-2 and family with sequence similarity 160 member B1), in the antisense miRNA-222-transfected MCF7-FR cells. The miRNA-target regulatory network is represented in Fig. 2.

Discussion

In the present study, 492 and 404 DEGs were identified in the antisense miRNA-221-transfected MCF7-FR cells and the antisense miRNA-222-transfected MCF7-FR cells, respectively, as compared with the negative control. GSEA revealed that the PPP was significantly enriched in the antisense miRNA-221-transfected and antisense miRNA-222-transfected MCF7-FR cells. Furthermore, 87 transcription factors were identified for the genes enriched in the PPP, which suggested that the PPP was significantly regulated in these cells. The PPP produces two substrates, ribose 5-phosphate and nicotinamide adenine dinucleotide phosphate, which are necessary for the division of cells and serve as buffers to prevent reactive oxygen species-induced cell death and apoptosis (17). Alterations in the PPP activity have been reported to occur during cancer development and progression (18). In addition, an increase in the levels of various PPP metabolites in the breast epithelia, including sedoheptulose 7-phosphate and hexose phosphate intermediates, has been reported to occur during the transition from normal breast epithelial cells to transformed cells, as well as during the transition from non-metastatic to metastatic tumors (19,20).

In the present study, the Wnt signaling pathway was significantly enriched in the antisense miRNA-222-transfected MCF7-FR cells compared with the normal control-transfected cells. A total of 94 transcription factors were associated with the genes enriched in the Wnt signaling pathway, which suggested that this pathway was highly regulated in the miRNA-222-transfected MCF7-FR cells. A previous study reported that the activation of the Wnt signaling pathway could lead to the metastasis of breast cancer (21). In addition, the blockage of Wnt signaling has been demonstrated to inhibit cell proliferation and migration, and to induce apoptosis in triple-negative breast cancer cells (22).

In the present study, the CAMs pathway was significantly enriched in the antisense miRNA-222-transfected MCF7-FR cells compared with the normal control-transfected cells. A total of 123 transcription factors were associated with the genes enriched in this pathway. CAMs are membrane receptors that mediate cell-cell and cell-matrix interactions, and have an essential role in transducing intracellular signals responsible for adhesion, migration, invasion, angiogenesis and organ-specific metastasis (23). Adhesion molecules, including E-cadherin and carcinoembryonic antigen, have been associated with the process of metastasis in breast cancer cells (24). Taken together, these results suggested that the PPP, Wnt signaling pathway and CAMs pathway may be associated with the resistance of breast cancer to fulvestrant.

In the miRNA-target regulatory network, miR-222 was observed to target *PCDH10*. *PCDH10* is a member of the mammalian cadherin superfamily, which has key roles in cell migration and calcium-dependent, cadherin-mediated homophilic cell-cell interactions (25). A previous study identified *PCDH10* as a candidate tumor suppressor in nasopharyngeal, esophageal and various other carcinomas, in which it was associated with frequent methylation (26). As a result, *PCDH10* targeted by miR-222 could be associated with the resistance of breast cancer to fulvestrant.

In conclusion, the results of the present study suggested that the PPP, Wnt signaling pathway and CAMs KEGG pathway, as well as *PCDH10*, may be associated with the development of fulvestrant resistance in patients with breast cancer. However, further studies are required to elucidate the underlying mechanisms.

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