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ORIGINAL ARTICLE

Evidence for the role of microRNA 374b in acquired cisplatin resistance in pancreatic cancer cells

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Recent evidence has implicated microRNAs (miRNAs) as potentially significant players in the acquisition of cancer-drug resistance in pancreatic and other cancers. To evaluate the potential contribution of miRNAs in acquired resistance to cisplatin in pancreatic cancer, we compared levels of more than 2000 human miRNAs in a cisplatin-resistant cell line (BxPC3-R) derived from parental (BxPC3) cells by step-wise exposure to increasing concentrations of the drug over more than 20 passages. The acquired drug resistance was accompanied by significant changes in the expression of 57 miRNAs, of which 23 were downregulated and 34 were upregulated. Employing a hidden Markov model (HMM) algorithm, we identified downregulation of miR-374b as likely being directly involved in acquisition of the drug-resistant phenotype. Consistent with this prediction, ectopic overexpression of miR-374b in the resistant BxPC3-R cells restored cisplatin sensitivity to levels approaching those displayed by the BxPC3 parental cells. The results are consistent with a growing body of evidence implicating miRNAs in acquired cancer-drug resistance and with the potential therapeutic value of these small regulatory RNAs in blocking and/or reversing the process.

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INTRODUCTION

The ability of cancer cells to acquire resistance to chemotherapy is one of the most pressing and challenging issues in contemporary clinical oncology.[1] The problem is especially acute for pancreatic cancer where tumors are unresectable in over 80% of patients making radio/chemotherapy the only viable alternatives.[2] Recent studies in pancreatic[3–5] and other cancers[6,7] have identified microRNAs (miRNAs) as potentially important regulatory elements underlying coordinated changes in gene expression associated with acquired drug resistance. As such, miRNAs have been proposed as a potential new class of agents for targeted treatment of acquired drug resistance.[8–10]

We report here evidence for the contribution of miRNAs in the acquisition of cisplatin resistance in a pancreatic cell line (BxPC3-R) developed by step-wise increasing concentrations of the drug over more than 20 passages.[11] Using a hidden Markov model (HMM) algorithm to find miRNAs most likely contributing to gene expression changes associated with cisplatin resistance in BxPC3-R cells, we identified downregulation of miR-374b as putatively involved in acquisition of the drug-resistant phenotype. Consistent with this prediction, ectopic overexpression of miR-374b in the resistant BxPC3-R cells restored cisplatin sensitivity to levels approaching those displayed by the BxPC3 parental cells. Our results are consistent with the growing body of evidence indicating that changes in miRNA levels can have a significant role in the acquired resistance of cancer cells to therapeutic drugs and that therapies designed to modulate levels of these small regulatory RNAs may be of significant therapeutic value in blocking and/or reversing acquired drug resistance.

MATERIALS AND METHODS

Cell culture

The cisplatin-resistant pancreatic cancer cell line BxPC3-R was developed from parental human pancreatic adenocarcinoma BxPC3 cell line (ATCC CRL-1687) by step-wise treatment as previously described.[11] Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Parental cells were maintained in RPMI-1640 (Mediatech, Manassas, VA) supplemented with 10% FBS (fetal bovine serum; Atlanta Biologicals, Lawrenceville, GA) and 1% antibiotic-antimycotic solution (Mediatech). Cisplatin-resistant cells were routinely maintained in the full RPMI medium supplemented with 0.6 μM cisplatin. Before harvesting for experiments, BxPC3-R cells were grown 1 × in cisplatin-free medium.

Growth inhibition assay

The growth inhibitory effects of cisplatin on the BxPC3 and BxPC3-R were determined by measuring cell viability using the TOX-8 reagent (Resazurin based *in vitro* toxicology assay kit, Sigma-Aldrich, St Louis, MO). Cells were plated in 100 μl media on 96-well plates at a density of 3000 cells per well. Subsequent to 24 h incubation, the cells were exposed to different concentrations of cisplatin in total volume of 200 μl per well at 37 °C under a 5% CO_2 atmosphere for 72 h. Tox-8 (20 μl) was then added to each well; incubation continued for an additional 4 h and fluorescence was read using the Synergy 4 (Biotek, Winooski, VT) microplate reader ($\lambda_{ex} = 560$ nm, $\lambda_{em} = 590$ nm). Blank-corrected fluorescence signals for treated cells were normalized to control wells (no drug treatment) and expressed as a percentage of the control (% cell viability). The results from three experiments were presented as mean ± s.e.m.

miRNA transfection

Transfection of the miR-374b mimetic and the negative control miRNA (both from Applied Biosystems, ThermoFisher Scientific, Grand Island, NY) at final concentrations of 30 nM was carried out using the Lipofectamine

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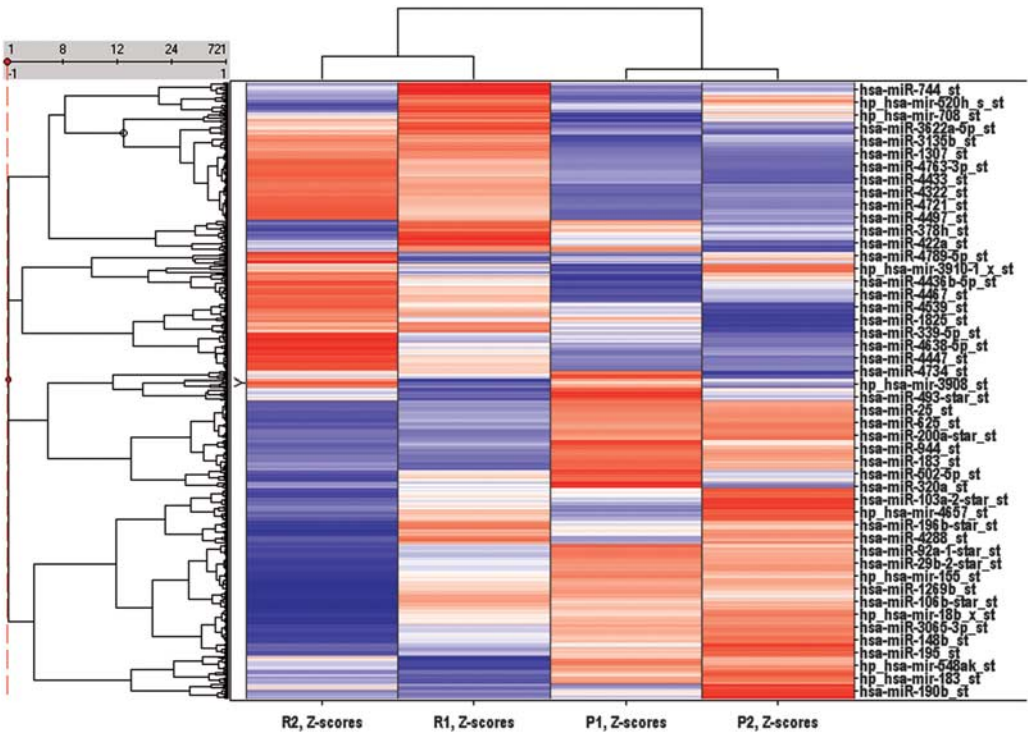

Figure 1. Hierarchical clustering of small RNA expression signals for BxPC3-R (R1, R2) and BxPC3 parental (P1, P2) cells. The image includes 721 annotated human microRNAs (miRNAs) that displayed at least one non-absent call in the processed microarray data. Distance method: Pearson; tree method: longest distance. Color-coding: blue = downregulated, white = not differentially expressed, red = upregulated, in BxPC3-R relative to BxPC3 cells. Due to space limitations, names of all miRNAs could not be shown.

2000 Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Cells were harvested for subsequent analyses 48 h after the transfection. All transfection experiments were carried out in triplicate.

RNA extraction, miRNA microarray analysis

Gene expression profiling of BxPC3 and BxPC3-R cells was performed using GeneChip miRNA 3.0 Array (Affymetrix, Santa Clara, CA). Cellular RNA enriched for small RNAs (<200 nt) was isolated using the mirVana miRNA isolation kit according to the manufacturer's instructions (ThermoFisher Scientific). The concentration and quality of miRNA were determined using the Small RNA Assay in the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). In all, 400 ng of cellular RNA enriched for small RNAs was labeled using the FlashTag Biotin HSR Labeling Kit, hybridized for 18 h at 48 °C (rotation 60 r.p.m.) on the GeneChip miRNA 3.0 Array, washed in the Affymetrix Fluidics Station 450 and scanned using the Affymetrix GeneChip Scanner 3000 7G (all from Affymetrix) as previously described.[12] Microarray experiments were performed in two replicates per each cell type. The data were analyzed with Expression Console software Build 1.2.1.20 (Affymetrix) using the default analysis setting for RMA+DAGB workflow and submitted to the Gene Expression Omnibus repository (GEO, available under the series accession number GSE79506).

Computational analysis

MiRNAs differentially expressed between parental BxPC3 and cisplatin-resistant BxPC3-R cells were identified by differential expression analysis of the normalized miRNA 3.0 Array data using the absolute fold change (FC) $\geqslant 2$ and ANOVA *P*-value <0.05 as a threshold.

The 561 annotated genes previously reported as upregulated in BxPC3-R cells relative to BxPC3 cells[11] (data are publicly available under Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; series accession number GSE73978) were uploaded to miRvestigator (http://mirvestigator.systemsbiology.net)[13] and analyzed for overrepresented sequence motifs to identify miRNAs most likely involved in the regulation of these genes through their complementary seeds. miRvestigator was employed using the following parameters: Weeder Parameters: Motif Sizes: 8 bp; Weeder Model: Default; Seed Models: 6mer, 7mer, 8mer; Wobble Base-Pairing: No. The top 10 miRNAs complementary to the overrepresented sequence motif were identified and compared with the list of miRNAs found to be differentially expressed between BxPC3 and BxPC3-R cells by miRNA microarray profiling.

RESULTS

Significant changes in the expression of miRNAs are associated with the acquisition of cisplatin resistance in BxPC3-R cells

Unsupervised hierarchical clustering of the expression profiles of miRNAs was carried out on two biological replicates each of the parental cisplatin-sensitive BxPC3 cells and derived resistant BxPC3-R cells (Figure 1). Using a threshold of $\geqslant 2$-FC, 57 miRNAs were identified as being significantly differentially expressed ($P<0.05$) between the BxPC3 and BxPC3-R cells. Of these, 23 miRNAs were downregulated and 34 were upregulated (Table 1).

Computational analysis of gene expression changes between BcPC3 and BcPC3-R cells implicates miR-374b in the acquisition of cisplatin resistance

We previously developed a pancreatic cancer cell line BxPC3-R with ~15-fold increase in resistance to cisplatin by exposing the well-characterized pancreatic adenocarcinoma cell line BxPC3 to stepwise increasing concentrations of the drug over more than 20 passages.[11] Comparative gene expression analysis determined that 1565 genes were differentially expressed between the cisplatin-resistant BxPC3-R cells relative to the pre-selected BxPC3 cells (Gene Expression Omnibus series accession number GSE73978).[11] Of these, 561 genes were significantly upregulated in the resistant BxPC3-R cells and many of these upregulated genes were found to have been previously associated with acquired drug resistance.[11]

In the current study, the 561 upregulated genes were uploaded to miRvestigator[13] to detect overrepresented sequence motifs

Table 1. miRNAs significantly differentially expressed between cisplatin-resistant BxPC3-R and parental BxPC3 cell lines (|FC| ⩾ 2 and ANOVA *P*-value < 0.05)

Transcript ID (array design)	*Accession*	*FC*	P-*value*
hsa-miR-7	MIMAT0000252	−3.76	0.011895
hsa-miR-126	MIMAT0000445	−3.41	0.023464
hsa-miR-374b	MIMAT0004955	−3.16	0.036522
hsa-miR-7-1-star	MIMAT0004553	−2.95	0.022411
hsa-miR-10a	MIMAT0000253	−2.76	0.010547
hsa-miR-98	MIMAT0000096	−2.73	0.015384
hsa-miR-21	MIMAT0000076	−2.6	0.000757
hsa-let-7f	MIMAT0000067	−2.5	0.010142
hsa-miR-23b-star	MIMAT0004587	−2.49	0.012496
hsa-miR-3907	MIMAT0018179	−2.48	0.013187
hsa-miR-196b	MIMAT0001080	−2.47	0.040264
hsa-miR-1293	MIMAT0005883	−2.44	0.00803
hsa-miR-584	MIMAT0003249	−2.33	0.000225
hsa-miR-20b-star	MIMAT0004752	−2.23	0.017333
hsa-miR-30c-2-star	MIMAT0004550	−2.17	0.038717
hsa-miR-183	MIMAT0000261	−2.16	0.014998
hsa-miR-1301	MIMAT0005797	−2.14	0.032281
hsa-miR-553	MIMAT0003216	−2.13	0.025518
hsa-miR-21-star	MIMAT0004494	−2.11	0.026038
hsa-miR-342-3p	MIMAT0000753	−2.08	0.027915
hsa-miR-26b	MIMAT0000083	−2.07	0.046819
hsa-miR-542-5p	MIMAT0003340	−2.07	0.018502
hsa-miR-30b-star	MIMAT0004589	−2.04	0.048326
hsa-miR-370	MIMAT0000722	2.01	0.048404
hsa-miR-149-star	MIMAT0004609	2.02	0.043553
hsa-miR-4651	MIMAT0019715	2.05	0.043143
hsa-miR-4687-3p	MIMAT0019775	2.05	0.043289
hsa-miR-4655-5p	MIMAT0019721	2.06	0.002981
hsa-miR-4322	MIMAT0016873	2.07	0.016038
hsa-miR-3656	MIMAT0018076	2.09	0.037679
hsa-miR-4741	MIMAT0019871	2.13	0.042265
hsa-miR-933	MIMAT0004976	2.13	0.014902
hsa-mir-933	MI0005755	2.15	0.032396
hsa-miR-1913	MIMAT0007888	2.2	0.024095
hsa-miR-4529-3p	MIMAT0019068	2.2	0.011137
hsa-mir-326	MI0000808	2.21	0.034926
hsa-miR-4739	MIMAT0019868	2.25	0.044837
hsa-miR-498	MIMAT0002824	2.34	0.010759
hsa-mir-4259	MI0015858	2.36	0.020057
hsa-miR-4706	MIMAT0019806	2.37	0.011136
hsa-miR-3141	MIMAT0015010	2.39	0.017538
hsa-miR-4649-5p	MIMAT0019711	2.46	0.047838
hsa-miR-4253	MIMAT0016882	2.48	0.042591
hsa-mir-4507	MI0016871	2.49	0.032776
hsa-miR-4449	MIMAT0018968	2.54	0.023089
hsa-miR-323-5p	MIMAT0004696	2.56	0.024994
hsa-miR-1224-5p	MIMAT0005458	2.84	0.015138
hsa-miR-1268b	MIMAT0018925	2.88	0.034795
hsa-miR-4689	MIMAT0019778	2.92	0.018471
hsa-miR-3162-5p	MIMAT0015036	2.96	0.049017
hsa-miR-4281	MIMAT0016907	3.12	0.039802
hsa-miR-4433	MIMAT0018949	3.46	0.029904
hsa-miR-551b-star	MIMAT0004794	3.65	0.00488
hsa-miR-1207-5p	MIMAT0005871	3.75	0.049755
hsa-miR-4685-5p	MIMAT0019771	4.15	0.024853
hsa-miR-658	MIMAT0003336	9.21	0.025669
hsa-miR-4525	MIMAT0019064	9.73	0.010276

Abbreviations: ANOVA, analysis of variance; FC, fold change.

within the genes' untranslated leader regions and to putatively identify those miRNAs most likely associated with their regulation. The miRvestigator algorithm identified a significantly overrepresented consensus sequence present among 31% of the upregulated gene sequences (5′-UAUUGUAA-3′) (Figure 2).

The 10 miRNAs identified by miRvestigator as containing sequences most significantly complementary to this consensus

Figure 2. Weeder motif identified by miRvestigator among 561 genes upregulated in BxPC3-R cells. The sequence shows the relative abundance of each nucleotide in the sequence based on the letter's height.

sequence are presented in Table 2. Of these, miR-374b was among the miRNAs most significantly downregulated in the BxPC3-R-resistant cells (FC = −3.16, Table 1). Moreover, miR-374b was found to have highly significant overall complementarity with the consensus sequence ($P < 7.3e-04$). Indeed, the consensus sequence is an exact compliment to the miR-374b 7-mer seed sequence (Table 2). Combined, these findings strongly implicated the downregulation of miR-374b in BxPC3-R cells with acquired cisplatin resistance.

Ectopic overexpression of miR-374b in cisplatin-resistant BxPC3-R cells decreases drug resistance to levels approaching those in pre-selected BxPC3 cells

To experimentally test the hypothesis that downregulation of miR-374b has contributed to acquired cisplatin resistance in BxPC3-R cells, we ectopically overexpressed an miR-374b mimetic in BxPC3-R cells and subsequently tested the sensitivity of the cells to increasing concentrations of cisplatin relative to controls.

The results presented in Figure 3 demonstrate that sensitivity to cisplatin was significantly increased in BxPC3-R cells in which miR-374b was ectopically overexpressed. The sensitivity of the miR-374b-transfected cells to cisplatin was not significantly different from the parental BxPC3 cells at the highest level of drug tested (4.5 μM) ($P = 0.25$). In contrast, cells transfected with the mock miRNA (negative control) maintained a level of cisplatin resistance statistically indistinguishable from the BxPC3-R-resistant cells. These results are consistent with the hypothesis that downregulation of miR-374b is a significant factor in the acquired cisplatin resistance of BxPC3-R cells.

DISCUSSION

Recent evidence has implicated miRNAs as potentially significant players in the acquisition of cancer-drug resistance in pancreatic[3–5] and many other cancer types.[6,7] In an effort to further evaluate the potential contribution of miRNAs in acquired drug resistance in pancreatic cancer, we compared levels of over 2000 human miRNAs in a well-characterized pancreatic cell line, BxPC3, and a cisplatin-resistant cell line (BxPC3-R) derived from BxPC3 cells by step-wise exposure to increasing concentrations of the drug over more than 20 passages.[11] We found that the acquired resistance of BxPC3 cells to cisplatin was accompanied by significant ($P < 0.01$) changes in the expression of 57 miRNAs, of which 23 were downregulated and 34 were upregulated. It is possible that many, if not all of these differentially expressed miRNAs, may have contributed either directly or indirectly to the acquisition of drug resistance in BxPC3-R cells. Indeed, several of the miRNAs differentially expressed between BxPC3-R and BxPC3 cells have been previously implicated in acquired drug resistance in pancreatic (for example, miR-7,[5] miR-21 (refs. 14,15) and

Table 2. Ten miRNAs predicted by miRvestigator to most likely target mRNAs overexpressed in cisplatin-resistant BxPC3-R cells

miRNA name	*miRNA seed*	*Seed model*	*Length of complementarity*	*Complementarity base-pairing*	*Complementarity P-value*
hsa-miR-374a	UUAUAAUA	8 mer	8	Motif 5′ UAUUGUAA 3′ 3′ AUAAUAUU 5′ miRNA seed	$1.2e^{-04}$
hsa-miR-4666-3p	UACAAU	6 mer_1	6	Motif 5′ UAUUGUAA 3′ 3′ UAACAU 5′ miRNA seed	$4.9e^{-04}$
hsa-miR-374b	UAUAAUA	7 mer_a1	7	Motif 5′ UAUUGUAA 3′ 3′ AUAAUAU 5′ miRNA seed	$7.3e^{-04}$
hsa-miR-338-5p	ACAAUA	6 mer_2	6	Motif 5′ UAUUGUAA 3′ 3′ UAAAAU 5′ miRNA seed	$7.3e^{-04}$
hsa-miR-3163	UAAAAU	6 mer_1	6	Motif 5′ UAUUGUAA 3′ 3′ UAAAAU 5′ miRNA seed	$1.2e^{-03}$
hsa-miR-4282	UAAAAU	6 mer_3	6	Motif 5′ UAUUGUAA 3′ 3′ UAAAAU 5′ miRNA seed	$1.2e^{-03}$
hsa-miR-600	ACUUACA	7 mer_m8	5	Motif 5′ UAUUGUAA 3′ 3′ ACAUUCA 5′ miRNA seed	$2.4e^{-03}$
hsa-miR-520d-5p hsa-miR-524-5p	UACAAA	6 mer_2	6	Motif 5′ UAUUGUAA 3′ 3′ AAACAU 5′ miRNA seed	$2.7e^{-03}$
hsa-miR-1283	UACAAA	6 mer_1	6	Motif 5′ UAUUGUAA 3′ 3′ AAACAU 5′ miRNA seed	$2.7e^{-03}$
hsa-miR-3613-3p	ACAAAA	6 mer_3	6	Motif 5′ UAUUGUAA 3′ 3′ AAAACA 5′ miRNA seed	$2.9e^{-03}$

miR-196a[5]) and other cancers (for example, miR-21,[16] miR-30c,[17] miR-122 (ref. 18) and miR-326 (ref. 19)).

Because experimental validation of the functional significance of each of the 57 differentially expressed miRNAs associated with acquired cisplatin resistance in BxPC3-R cells would be a monumental task beyond the scope of the present study, we sought a computational approach that could narrow focus to those most likely implicated in the regulation of the majority of genes differentially expressed between BxPC3 and BxPC3-R cells. miRvestigator is a HMM algorithm that systematically computes a similarity *P*-value for each unique miRNA seed sequence from the miRNA database to an overrepresented sequence motif identified within the 3′-UTR of the query genes.[13] In our case, the query genes applied to the algorithm were the 561 genes significantly upregulated in the BxPC3-R-resistant cells.

The miRvestigator algorithm identified the consensus sequence 5′-UAUUGUAA-3′ as being present within the untranslated leader regions of 31% of upregulated genes. This motif was identified as pairing significantly with the seed regions of a number of miRNAs including miR-374b, one of the most significantly downregulated miRNAs in BxPC3-R-resistant cells (Table 1).

The computational prediction that downregulation of miR-374b likely contributed to the acquisition of resistance to cisplatin in BxPC3-R cells was experimentally tested by transfection of miR-374b into BxPC3-R cells and subsequently measuring cisplatin sensitivity of these cells relative to controls. The results demonstrated that miR-374b transfection significantly reduced drug resistance in BxPC3-R cells to levels approaching those of the parental BxPC3 cells.

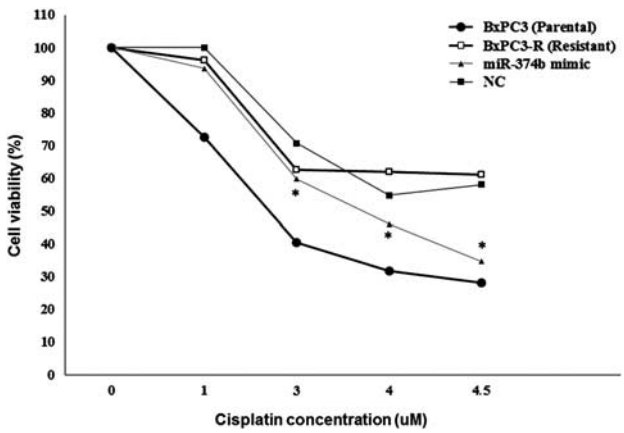

Figure 3. Effects of miR-374b mimetic or negative control microRNA (miRNA) on the sensitivity to cisplatin in BxPC3-R cells. Cells were treated with cisplatin for 72 h and cell viability relative to untreated cells was determined by Tox-8 reagent. Each data point represents the mean of three independent experiments (*$P < 0.05$, treated BxPC3-R cells relative to untreated BXPC3-R cells).

Mir-374b is predicted to directly target >7000 genes (http://www.microrna.org/) and downregulation of miR-374b, like most miRNAs, is predicted to result in upregulation of directly targeted genes.[20,21] Of the 561 genes upregulated in BxPC3-R cells, 201 are predicted to be directly targeted by miR-374b (Supplementary Table S1). Among these are genes previously implicated in cisplatin resistance including *ATP7A* (ATPase, Cu++ Transporting, Alpha Polypeptide)[22] and *CLU* (Clusterin).[23] This is not to say that these are the only genes likely involved in miR-374b-mediated acquisition of drug resistance nor that miR-374b is the only miRNA contributing to the process. Rather, emerging evidence indicates that drug resistance, like cancer onset and progression, is a system-wide process and not necessarily attributable to changes in the expression of one or a few genes.[24] Just as there are multiple molecular pathways involved in acquired drug resistance, there are likely to be multiple pathways by which drug sensitivity can be restored. The growing body of evidence for the involvement of miRNAs in acquired drug resistance[6–8,10,21] supports the systems view and identifies miRNAs as regulatory elements of potentially significant therapeutic value.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cancer Gene Therapy website (http://www.nature.com/cgt)