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Gene expression profiling of drug-resistant small cell lung cancer cells by combining microRNA and cDNA expression analysis

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ABSTRACT

MicroRNAs (miRNAs) are now known to play important roles in the regulation of gene expression for developmental timing, cell proliferation and apoptosis. Therefore, it is likely that they also modulate sensitivity and resistance to anti-cancer drugs. To better understand the molecular mechanisms of multidrug resistance in SCLC and identify novel molecular markers, we evaluated the expression of 856 miRNAs and ~22,000 genes using miRNA microarray and cDNA microarray in cellular models of SCLC which were widely used as sensitive (NCI-H69) and resistant cell lines (NCI-H69AR) to chemotherapy. We also analysed the correlations between miRNA and mRNA expression patterns. Further studies were tested to determine whether the differentially expressed miRNAs were involved in multidrug resistance in SCLC. Our results showed that 61 miRNAs are presented significantly (>3-fold) including up-regulation of 24 miRNAs and down-regulation of 37 miRNAs. Among these miRNAs, 48 of 61 differentially expressed miRNAs were firstly reported to be closely associated with drug resistance and 37.7% (24/61) of miRNA genes were organised as 10 clusters in total 61 significantly expressed miRNAs. We also found that only 27 of 69 miRNAs were significantly correlated with 604 of 21,522 70 mRNA transcripts by MAS database. The sensitivity to anti-cancer drugs Cisplatin, Etoposide and Doxorubicin greatly increased or reduced following transfection of the drug-resistant H69AR cells with the mimics or antagomirs of miR-134, miR-379 and miR-495, respectively. miR-134 increases the cell survival by inducing G1 arrest in H69AR cells. MRP1/ABCC1 is negatively regulated by miR-134 and down-regulation of MRP1/ABCC1 at the protein level largely correlates with elevated levels of miR-134 in H69AR cells. Our results support for the first time a substantial role for miRNAs in multidrug resistance in SCLC. miR-134 could be a causal factor of the down-regulation of MRP1/ABCC1 in H69AR cells. These findings provide valuable information for potential utility of these miRNAs as specific diagnostic biomarkers and novel therapeutic approaches for drug resistance of SCLC.

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1. Introduction

Lung cancer is one of the most common malignant tumours and the leading causes of cancer death throughout the world at present. Small cell lung cancer (SCLC) represents around 15% of all lung cancers.[1,2] Although SCLC is highly responsive to both chemotherapy and radiotherapy, it is characterised by high relapse rates and a subsequent poor prognosis because of drug resistance. The 2-year survival rate is generally less than 5% in patients with SCLC after final diagnosis.[3,4] Therefore, chemoresistance has become one of the major problems in the treatment of SCLC and is clinically a very important issue for improving poor prognosis of SCLC.

The molecular genetic basis of sensitivity and resistance to cancer therapeutics is complex, involving multiple processes such as drug transport, drug metabolism, DNA synthesis and repair, cell survival and apoptosis.[5–9] The novel molecular technologies such as genome-wide cDNA microarray analysis enabled us to obtain comprehensive gene expression profiles related to genes in chemoresistance. A substantial number of genes such as proapoptotic genes caspase (CASP3, 6), signal transcription factor NF-κB, mitogen-activated protein kinase 1, focal adhesion kinase/protein tyrosine kinase 2 (FAK/PTK2) and DNA-binding genes (C9orf76, EHD3) were identified to be associated to drug resistance to anti-cancer drugs.[10,11] A new class of potential genomic regulators which are now universally recognised as central players, named microRNAs.[12] MicroRNAs (miRNAs) are a class of small non-coding RNAs of 18–24 nucleotides which regulate protein expression of specific mRNA by either translational inhibition or mRNA degradation.[13–15] One strand of the mature double-stranded miRNA is incorporated into the RNA-induced silencing complex, which down regulates target mRNAs either by degradation or by translational inhibition.[14] miRNAs play important roles in the regulation of gene expression for developmental timing, cell proliferation and apoptosis. Because many of the same biological processes are relevant to cancer chemosensitivity and chemoresistance, miRNAs are therefore thought to be associated with drug sensitivity/resistance and the hypothesis has been proved in some cancer cells recently.[16–20] Mo et al. reported that miR-21 renders tumour cells insensitive to topotecan, a chemotherapy agent that acts as a topoisomerase I inhibitor, and anti-miR-21 oligonucleotide could enhance the inhibitory effect of topotecan on growth of tumour cells.[16] Alteration of the miR-24-binding site in dihydrofolate reductase gene results in resistance to a commonly used anti-cancer agent methotrexate.[17] Up-regulation of miR-214 promotes survival of ovarian cancer cells and induces resistance to Cisplatin.[18] Down-regulation of miR-27a or miR-451 increased the sensitivity of ovarian cancer cells to cytotoxic drug vinblastine.[19] Suppression of miR-21 expression in a cholangiocarcinoma cell line increased the sensitivity to gemcitabine.[20] Nevertheless, whether or not miRNAs are involved in the drug resistance of SCLC cells has not yet been reported.

Up to 30% of the genes in mammalian genomes have been predicted to be regulated by miRNAs.[21,22] Therefore, miRNA regulation is highly likely to underlie many of the differential expression patterns being observed. A number of computational algorithms have been developed to predict the targeting of a given mRNA by a specific miRNA. Some of the more popular prediction algorithms including PicTar, TargetScan and miRanda are used to look up predicted miRNA: mRNA functional pairs for a specific miRNA or gene of interest.[22–24] However, Wang et al. more recently demonstrated that the majority of the correlation coefficients are not statistically significant possibly due to that the true positive discovery rate of the two prediction programs cannot be accurately estimated in spite of the constant improvement of the algorithms.[25]

To better understand the molecular mechanisms of multidrug resistance in SCLC, we evaluated the expression of 856 miRNAs and ~22,000 genes using miRNA microarray and cDNA microarray in cellular models of SCLC which were widely used as sensitive (NCI-H69) and resistant cell lines (NCI-H69AR) to chemotherapy. We also analysed the correlations between miRNA and mRNA expression patterns. Further studies were tested to determine whether the differentially expressed miRNAs were involved in multidrug resistance in SCLC.

2. Materials and methods

2.1. Cell culture

The human small lung cancer cell line NCI-H69 and the drug-resistant subline H69AR were purchased from the American Type Culture Collection (ATCC, USA). The H69 cell line was originally obtained from the pleural fluid of an untreated male patient diagnosed with SCLC. Cancer cells were then grown in suspension as cell aggregates. The H69AR, grown as a standard monolayer culture, was derived from H69 and showed resistance to topoisomerase II, an inhibitor (Doxorubicin). All cells were maintained in RPMI 1640 medium containing L-glutamine with 10% foetal calf serum in an incubator at 37 °C with 5% CO_2. H69AR was alternately fed with drug-free medium and medium containing 0.8 μM of Adriamycin (ADM), and overexpresses MRP1/ABCC1.[26–28] The resistant cell line was tested regularly for maintained resistance to the selected drugs. Growth and morphology of all cell lines were monitored on a weekly basis.

2.2. RNA extraction and reverse transcription

Total RNA was prepared by using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. The RNA was assessed by denaturing using agarose gel electrophoresis (visual presence of sharp 28S and 18S bands) and was quantitated by spectrophotometry. Two micrograms of total RNA from each sample were reverse transcribed with the cDNA Synthesis Kit (TaKaRa, China) and cRNA was produced by *in vitro* transcription (IVT) by T7 RNA polymerase using T7 RiboMAX Express Large Scale RNA Production System (Promega). cRNA (2 mg) was reversely transcribed by Superscript II reverse transcriptase and 9-nt random primer.

2.3. miRNA microarray

The same total RNA samples used for mRNA microarray analysis were also used for the miRNA microarray analysis. The

RNAs were labelled and then hybridised to a microarray chip with multiple repeat regions and a miRNA probe region, which detects miRNA transcripts listed in Sanger miRBase version 12.0 consisting of 856 human miRNA sequences plus two control probes (LC Sciences, Houston, TX, USA). Hybridisation used 100 μl 6 × SSPE buffer (0.90 M NaCl, 60 mM Na_2H-PO_4, 6 mM EDTA, pH 6.8) containing 25% formamide at 34 °C. The microarrays were scanned in a GenePix 4000B scanner (Axon Instruments, Union City, CA, USA) at wavelengths 635 and 532 nm for Cy5 and Cy3 dyes, respectively.

2.4. cDNA expression array

Human genome oligo array (22 K) was designed by CapitalBio Corporation (Beijing, China). CapitalBio 22 K Human Genome Oligo Array comprises 21,522 70-mer oligo probes, each representing one transcript of the human genome.[29] RNA samples as prepared above were labelled with Cy5 (red) and Cy3 (green) dyes, respectively. Labelled samples were then hybridised to array gene chips. Arrays were scanned using CapitalBio's confocal scanner LuxScan 10K-A (Beijing, China) and the obtained images were analysed with SpotData software (CapitalBio). An intensity-dependent lowess program in the R language package was used to normalise the two channel ratio values.

2.5. Real-time quantitative RT-PCR of miRNA and mRNA

Validation of differential gene expression was performed for a number of genes that were differentially expressed between H69AR and H69. The mRNA and mature miRNAs were amplified as previously described.[30] A FastStart DNA Master SYBR green I kit and a LightCycler (both from Roche Diagnostics) were used, following the manufacturer's protocols. Bulge-loop™ miRNA qPCR primers for each miRNA were synthesised by Ribobio (Guangzhou, China). A panel of PCR primers (Table 1) specific to the 16 representative genes that were up-regulated and down-regulated were synthesised by Invitrogen (Shanghai, China). The 10-μl PCR reaction contained 1 μl RT product, 1X PCR Master Mix, 15 nmol/l forward primer and 15 nmol/l reverse primer. The thermal profile for the real-time PCR was at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 35 s and 72 °C for 3 s on a Bio-Rad CFX96 real-time PCR system. All quantitative PCR reactions, including no-template controls, were performed in triplicate. Expression levels of each miRNA or mRNA were evaluated using comparative threshold cycle (Ct) method as normalised to that of U6 ($2^{-\Delta Ct}$). The fold changes of each miRNA or mRNA were calculated from the expression levels between H69AR cells and H69 cells.

2.6. RT-PCR

We selected ABCC1 which overexpresses in H69AR, and examined its expression levels by means of RT-PCR. Primers RT-PCR were designed using Primer Express software version 2.0 (Applied Biosystems) and synthesised by Invitrogen. The sequences of the ABCC1 primer was 5′-ATGTCACGTGGAATACCAGC-3′ (forward), 5′-GAAGACTGAACTCCCTTCCT-3′ (reverse). Amplification of GAPDH cDNA served as an internal control. PCR amplification was performed at an annealing temperature of 55 °C for 28 cycles (ABCC1) or 25 cycles (GAPDH), respectively. The PCR products were analysed by electrophoresis in 2% agarose containing ethidium bromide.

2.7. miRNA transfection

Cells were plated in 96-well plates at 1×10^4 cells/well and cultured for 16 h in 200 μl of serum-free, antibiotic-free, medium and then transfected with the mimics or antagomirs of miR-134 cluster including miR-134, miR-379, miR-382, miR-494 and miR-495, or negative miRNA control mimic or antagomir (100 pmol) (Ribobio, Guangzhou, China) using ligofectamine 2000 and OPTI-MEM I (Invitrogen), according to the manufacturer's protocol. The effects of mimics or antagomirs of miRNAs on *in vitro* drug cytotoxicity were examined at 24 h following transfection. The treatment was done in triplicate.

2.8. Northern blot analysis

Total RNA (~20 μg) was separated on a 15% Urea–PAGE gel and transferred to a nylon membrane. The membrane was prehybridised at 42 °C for 45 min and then hybridised with ^{32}P-labelled probes for miR-134 in combination with a ^{32}P-labelled

Table 1 – Representative gene primers used to perform quantitative PCR of mRNA.

Gene	Primer sequences (5′–3′)
ABCC1	Forward: ATGTCACGTGGAATACCAGC
	Reverse GAAGACTGAACTCCCTTCCT
EphA2	Forward: CCCCGCCCCTAGTTAGAGG
	Reverse: GAAAGCAAGAAGCTGGCCC
FZD1	Forward: GTGAGCCGACCAAGGTGTAT
	Reverse: AGCACAGCACTGACCAAATG
DLL1	Forward: CCTACTGCACAGAGCCGATCT
	Reverse: GCAGGTGGCTCCATTCTTGC
Jagged 1	Forward: CGGGATTTGGTTAATGGTTATC
	Reverse: ATAGTCACTGGCACGGTTGTAGCAC
Hox-1F	Forward: AGTTGGAGAGTACGGCTACCTG
	Reverse: TGCAGGGATGCAGCGATCTCCAC
GLI-1	Forward: TGGATATGATGGTTGGCAAGTG
	Reverse: ACAGACTCAGGCTCAGGCTTCT
IGFBP-7	Forward: GCGAGCAAGGTCCTTCCATA
	Reverse: GGGATTCCGATGACCTCACA
TNFRSF12A	Forward: CCTGCCCACTTCAGGATGCTA
	Reverse: CCTCACTGGATCAGTGCCACA
SNAI2	Forward: CTACAGCGAACTGGACACACA
	Reverse: TTGTGGTATGACAGGCATGG
ANXA1	Forward: AGGGTGACCGATCTGAGGAC
	Reverse: CTGGTGGTAAGGATGGTATTGA
CTNND2	Forward: AGCAGTTTCAGTGGCAGTCA
	Reverse: TCCTGTGGGTCAAGTATTCCT
SCGN	Forward: AGAAGTGGATGGGTTTGTC
	Reverse: CTGGGATTATGGGTTGATT
TGFBI	Forward: TGTGGCAAATCAACAGTCAT
	Reverse: CACAGTTCACAGTTACAATCCC
BAG2	Forward: CTTTGAGAGAAGCAGCAACTG
	Reverse: TGACACTTCAACGGTGAGAG
CACNA2D2	Forward: GACTTCATCGAGGACCCAAA
	Reverse: CGATGGAAGGGATCTCAAAA
β-Actin	Forward: GAGGTGATAGCATTGCTTTCG
	Reverse: CAAGTCAGTGTACAGGTAAGC

U6 RNA probe at 35 °C in 7% SDS/0.2 M Na_2PO_4 (pH 7.0) overnight. After being washed twice with 2 × SSPE [standard saline phosphate/EDTA (0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA)]/0.1% SDS and twice with 0.5 × SSPE/0.1% SDS, the membrane was exposed to x-ray film. The signal was quantified by gel scanning. The membrane was stripped by boiling in 0.1% aqueous SDS/0.1 × SSC for 10 min and rehybridised twice.

2.9. *In vitro drug resistance assay*

In vitro drug cytotoxicity was measured by Cell Counting Kit-8 (CCK-8) assay. The cells incubated without drugs (i.e. control wells) were set at 100% survival and were used to calculate the concentration of each cytostatic drug lethal to 50% of the cells (IC_{50}). The ranges of drug concentrations were based on earlier studies (no publication data) and aimed at obtaining an LC_{50} value for both highly sensitive and resistant cases. A total of three anti-cancer drugs [Cisplatin (DDP; Shangdong, China), Etoposide (VP-16; Jiangshu, China) and Doxorubicin (DOX; Guangdong, China)], obtained from commercial sources, were dissolved according to the manufacturer's instructions and tested in five concentrations. Cell death induced by anti-cancer drugs was quantified using the CCK-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt] assay. The Cells were seeded into 96-well plates (5000 cells/well) and then treated for 12 h in 200 μl of medium with anti-cancer drugs. CCK-8 reagent (Dojindo, Kumamoto, Japan) was then added and the cells were incubated at 37 °C for 4 h before reading the absorbency using a micro-plate reader (μQuant, Bio-Tek Instruments, Inc.) at 450 nm. The assay was conducted in six replicate wells for each sample and three parallel experiments were performed.

2.10. *Cell cycle analysis*

For cell cycle analysis by flow cytometry, H69AR cells (1×10^6) were transfected with miR-134 mimic or antagomir or negative control in six-well plates for 24 h as described above. The cells were washed twice in cold PBS buffer and fixed with 70% ethanol. After centrifugation at 2000*g* for 5 min, the cells were then treated with 0.1 mg/ml RNase A (Boehringer Mannheim) and stained with 50 mg/ml propidium iodide (Sigma Chemical). All samples were prepared following the manufacturer's instructions and analysed by flow cytometry (FACSCalibur, Becton Dickinson) using CellQuest software (BD Bioscience).

2.11. *Data and statistical analyses*

The raw microarray data set was filtered according to a standard procedure to exclude spots with minimum intensity that was arbitrarily set to an intensity parameter of $\geqslant$300 for cDNA expression data and $\geqslant$100 for the miRNA microarray data in both fluorescence channels. If the fluorescence intensity of one channel was below the cut-off while the other was above, the lower channel intensity was overridden. Spots with diameters <25 μm for cDNA expression array and <10 μm for miRNA microarry and flagged spots were also excluded from the analyses. For two colour experiments, the ratio of the two sets of detected signals (log_2 transformed, balanced) and *p*-values of the *t*-test were calculated; differentially detected signals were those with less than 0.01 *p*-values.

2.12. *Bioinformatics integration of mRNA and miRNA expression data*

miRNA target prediction in this study was performed by a molecule annotation system (MAS, http://bioinfo.capitalbio.com/mas/) software (CapitalBio). The primary database of MAS integrates numerous high-content biological resources from public knowledgebase including Genbank, EMBL, SwissProt, Gene Ontology (GO), KEGG, BioCarta, GenMapp, miRBase, EPD, HPRD, MIND, BIND, IntAct,TRANSFAC, UniGene, dbSNP, OMIM, InterPro, HUGO, MGI and RGD, with timely upgrades of a wide range of knowledgebase accessions. miRNAs and mRNAs expression data were integrated into a higher function relational data set called the 'Transcriptome Interaction Database' by MAS and by additional manual annotation.

3. Results

3.1. *miRNA expression profiling*

A microarray platform optimised for the analysis of a panel of 856 human miRNAs was used to analyse and compare the pattern of miRNA expression between parental SCLC cell line H69 and its counterpart drug-resistant cell line H69AR. The expression profiles of 61 miRNAs changed significantly (3.0–256-fold) including 24 up-regulated miRNAs and 37 down-regulated

Table 2 – Paralogous groups of miRNA clusters in H69AR cells (<10 kb).

Cluster	Change	Mapping	miRNA(fold change, log_2)
134-Cluster	Down	14q32.31	miR-134(–3.93); miR-299(–3.81); miR-329(–3.05); miR-376a(–4.66); miR-376c(–4.26); miR-379(–4.70); miR-382(–4.86); miR-409(–2.58); miR-485(–4.15); miR-487b(–5.26); miR-494(–3.88); miR-495(–4.51); miR-654(–3.89)
143-Cluster	Up	5q32	miR-143(7.41); miR-145(7.06)
127-Cluster	Down	14q32.2	miR-127(–4.21); miR-432(–5.65)
195-Cluster	Up	17p13.1	miR-195(4.78); miR-324(1.69)
216-Cluster	Down	2p16.1	miR-216a(–5.33); miR-217(–5.89)
99b-Cluster	Up	19q13.41	miR-99b(2.11); miR-125a(2.10)

miRNAs in H69AR cells as compared to H69 cells. We further analysed the clustering properties of all 61 differentially expressed miRNAs and found that 24 miRNA genes are organised in a total of six clusters, implying that 37.7% of the miRNAs are clustered (Table 2).

To confirm the results obtained by microarray analysis, we arbitrarily selected 14 miRNAs changed significantly in the ratio of H69AR/H69 and quantified their expression by real-time PCR. Table 4 summarises the miRNA measurements of all validated genes. We found that both methods (microarray analysis and real-time PCR) detected similar patterns for the seven up-regulated and seven down-regulated genes selected for validation.

3.2. *mRNA expression profiling*

A cDNA microarray analysis was performed to investigate the expression profiles of the 21,522 genes in H69AR cells and parent H69 cells. Of these genes, a total of 1252 genes were listed up as commonly up-regulated and 1131 genes as commonly down-regulated in H69AR (representative genes are listed in Table 3). The up-regulated and down-regulated genes represented a variety of functions including genes associated within chemosensitivity, cell proliferation and cell cycle control, apoptosis, cytoskeleton and signal transduction (Table 3). H69AR showed an increased expression of ABCCl compared to all other cell lines, which is consistent with previous results, further supporting the validity of the array measurements.[31]

To further validate microarray results, we performed real-time PCR experiments for 16 representative genes, which were indicated to be overexpressed commonly in SCLC cells. The results of RT-PCR experiments were quite concordant with those of the microarray data (Table 4).

3.3. *Correlation between miRNA and mRNA expression profiles*

miRNAs regulate gene expression by targeting mRNAs in a sequence-specific manner, inducing translational repression or mRNA degradation, depending on the degree of complementarity between miRNAs and their targets.[32] We combined our miRNA expression data and mRNA expression data to generate a miRNA-mRNA interaction database by

Table 3 – List of selected differentially expressed genes in H69AR and H69 cells.

Gene name	Description	Fold change	Functions
Vim	Vimentin	82.95	Cytoskeleton
TGFBI	Transforming growth factor-beta-induced protein IG-H3 precursor	46.38	Extracellular matrix
IGFBP-7	Insulin-like growth factor-binding protein 7 precursor	38.45	Cell proliferation
LGALS1	Lactose-binding lectin 1	36.22	Cell apoptosis
Wnt-3	Wnt-3 proto-oncogene protein precursor	20.46	Extracellular matrix
ARHGAP6	Rho-GTPase-activating protein 6	17.89	Small GTPase-mediated signal transduction
EphA3	Ephrin type-A receptor 3 precursor	16.29	Enzyme-linked receptor protein-signalling pathway
ANXA1	Annexin A1	15.19	Ion channel activity, cell apoptosis, proliferation
IGF1	Insulin-like growth factor IA precursor	11.42	Cell proliferation
FOXC1	Forkhead box protein C1	10.12	Transcription factors
BAG2	BAG-family molecular chaperone regulator-2	9.61	Cell apoptosis
FZD1	Frizzled 1 precursor	9.19	Wnt receptor signalling
PDGFC	Platelet-derived growth factor C precursor	7.90	Cell proliferation
Jagged 1	Jagged 1 precursor	7.14	Notch signalling
FRZB	Frizzled-related protein precursor	6.88	Wnt receptor signalling
CDKN2D	Cyclin-dependent kinase 4 inhibitor D	−3.85	Cell proliferation
SULT1E1	Sulphotransferase, oestrogen-preferring	−6.25	Drug metabolism
GCA	Grancalcin ion channel activity	−6.67	Ion channel activity
NALP2	PYRIN-containing APAF1-like protein 2	−8.33	Cell apoptosis
CDKN1A	Cyclin-dependent kinase inhibitor 1A	−9.09	Cell proliferation
CTNND2	Catenin delta-2	−9.09	Cytoskeleton
CACNA2D2	Alpha 2 delta calcium channel subunit	−9.09	Calcium channel activity, cell apoptosis
ELMO1	Engulfment and cell motility protein 1	−11.11	Cell apoptosis
PTPRD	Protein-tyrosine phosphatase delta precursor	−11.11	Enzyme-linked receptor protein-signalling pathway
UNC5A	UNC5A protein	−14.29	Cell apoptosis
DACT1	Dapper homologue 1	−14.29	Wnt receptor signalling
DLL1	Delta-like protein 1 precursor	−20.00	Notch signalling
SNAI2	Zinc finger protein SLUG	−20.00	Transcription factor
S100A14 S100	Calcium-binding protein A14	−20.00	Cell proliferation
SCGN	Secretagogin	−50.00	Calcium flux, cell proliferation
FABP7	Fatty acid-binding protein	−50.00	Cell proliferation

Table 4 – Ratios of differentially expressed miRNAs and mRNAs in H69AR and H69 cells.

miRNA	Fold change (H69AR/H69)		mRNA	Fold change (H69AR/H69)	
	Microarrays (log_2)	qRT-PCR		Microarrays (log_2)	qRT-PCR
miR-375	−8.31	−108.50	TGFBI	46.38	47.84
miR-200b	−8.19	−22.42	IGFBP-7	38.45	57.63
miR-335	−8.04	−7.28	TNFRSF12A	16.37	17.63
miR-217	−5.89	−3.57	ANXA1	15.19	12.59
miR-379	−4.70	−8.58	BAG2	9.61	10.25
miR-495	−4.51	−92.46	FZD1	9.19	7.66
miR-134	−3.93	−3.87	EphA2	7.85	7.14
miR-224	7.70	110.99	Jagged 1	7.14	15.45
miR-143	7.41	2.43	GLI-1	4.34	6.75
miR-145	7.06	15.23	ABCC1	2.60	28.48
miR-100	6.64	202.51	Hox-1F	−4.16	−9.09
miR-99a	5.21	113.27	CTNND2	−9.09	−8.33
miR-214	5.02	24.00	CACNA2D2	−9.09	−10.78
miR-29a	2.61	2.59	SNAI2	−20.00	−53.00
			DLL1	−20.00	−55.00
			SCGN	−50.00	−65.00

MAS software (Table 5). For example, 41 mRNAs may be the putative targets of miR-134-cluster which consists of miR-134, miR-379, miR-382, miR-494 and miR-495. ARHGDIB expression which is a substrate of caspase-3 and closely associated with apoptosis may be mediated by miR-134. miR-379 is likely to be involved in transcriptional regulation of TADA2L and MAP4K5 in the assembly of transactivation-competent beta-catenin complexes at Wnt target genes or in MAPK pathways. miR-495 was down-regulated and was predicted to up-regulate and down-regulate several genes. For example, ARHE (Rho-related GTP-binding protein RhoE) belongs to the Rnd subfamily of small Rho-related GTP-binding proteins which regulates actin cytoskeleton dynamics.[33] ARHE was up-regulated by 2.33-fold. miR-375 was down-regulated by over 256-fold and may be closely associated with glycosyltransferase-like 1, guanine nucleotide-binding protein G (GNAZ), ADP-ribosylation factor-like protein 7 (ARL7), Neural cell adhesion molecule1 (NCAM1), Bone morphogenetic protein 7 precursor (BMP-7) and heat shock 70 kDa protein 12A (HSPA12A) expression among thousand predicted genes in H69AR cells. TP53 apoptosis effector (PERP) was down-regulated by 4.6-fold, and BAG2 was up-regulated by 9.6-fold, potentially as results of miR-200b. The highly up-regulated miR-224 by 128-fold was supposed to be involved in the down-regulation of genes related with cell proliferation, such as cyclin-dependent kinase 4 inhibitor D (p19-INK4d) and cyclin-dependent kinase inhibitor 1 (p21). Similarly, up-regulated miR-195 may inhibit DLL1 expression by the notch-signalling pathway.

3.4. *miRNAs modulate cell survival and multidrug resistance*

We further determined whether inhibition or increase of miR-134 cluster including miR-134, miR-379 and miR-495 could modulate cell survival and the sensitivity of H69AR cells to chemotherapeutic drugs Cisplatin, Etoposide and Doxorubicin, all of which are currently used for SCLC chemotherapy. Following transfection of the H69AR cells with the mimics of miR-134, miR-379 and miR-495, respectively, we treated the cells with a series of concentrations of Cisplatin, Etoposide and Doxorubicin. The effect of the mimics on sensitivity to these three chemotherapeutic drugs was significantly increased by 2.79–5.17-fold in H69AR cells (Fig. 1). Meanwhile, the growth inhibition by chemotherapeutic drugs in H69AR cells was 1.37–1.53-fold greater in the antagomir-treated cells than that in control cells (Fig. 2).

In order to confirm the effects of mimics and antagomirs on miRNA expression, we selectively analysed miR-134 expression with Northern blot. As shown in Fig. 3, miR-134 was up-regulated following transfection of the H69AR cells with miR-134 mimic, while it was down-regulated in the cells with miR-134 antagomir as compared with the control.

3.5. *Effect of miR-134 on cell cycle control*

To determine the impact of miR-134 on cell cycle control, we analysed the cell cycle by flow cytometry using H69AR cells transfected with miR-134 antagomir and mimic. The results showed that miR-134-induced G1 (64.6%) arrest following transfection with miR-134 antagomir (Fig. 4b) by contrast with the control (49.7%) (Fig. 4a). The results in miR-134 mimic-transfected cells were consistent with those in miR-134 antagomir-transfected cells (Fig. 4c).

3.6. *miR-134 targets MRP1/ABCC1 in drug-resistant cells*

MRP1/ABCC1, a 190 kDa transmembrane glycoprotein that belongs to the ATP-binding cassette superfamily, has been reported to overexpress in drug-resistant subline H69AR.[26–28] We therefore investigated whether miR-134 can regulate MRP1/ABCC1 expression by RT-PCR and Western blotting although MRP1/ABCC1 was not the putative target by MAS software. The results revealed that MRP1/ABCC1 protein but not mRNA was considerably decreased in miR-134 mimic-transfected cells (Figs. 5 and 6). In contrast, there was a small

Table 5 – Hypothetical targets of several miRNAs in H69AR.

miRNA	mRNA name	Description
miR-375	NP_078935	Glycosyltransferase-like 1
	GNAZ	Guanine nucleotide-binding protein G(z)
	ARL7	ADP-ribosylation factor-like protein 7
	NCAM1	Neural cell adhesion molecule 1
	BMP7	Bone morphogenetic protein 7 precursor (BMP-7)
	HSPA12A	Heat shock 70 kDa protein 12A
miR-200b	PERP	TP53 apoptosis effector
	BAG2	BCL2-associated athanogene 2
	ZFHX1B	Zinc finger homeobox protein 1b
miR-134	NP_443131	G protein-regulated inducer of neurite outgrowth 1
	LTB	Lymphotoxin-beta (LT-beta) (tumour necrosis factor C) (TNF-C)
	ARHGDIB	Rho GDP-dissociation inhibitor 2 (Rho GDI 2)
miR-379	MAP4K5	Mitogen-activated protein kinase kinase kinase kinase 5
	KCNJ3	G protein-activated inward rectifier potassium channel 1
	TADA2L	Transcriptional adapter 2-like (ADA2-like protein)
	ST18	Suppression of tumourigenicity 18; zinc finger protein 387
miR-382	VIM	Vimentin
	PDGFRL	Platelet-derived growth factor receptor-like protein
	CHST9	GalNAc-4-sulphotransferase 2
	PTD4	Putative GTP-binding protein
	SMOC1	SPARC-related modular calcium-binding protein 1 precursor
	GPR160	Probable G protein-coupled receptor 160
miR-494	ZIC2	Zinc finger protein ZIC 2
	SCGN	Secretagogin
	SLC25A13	Calcium-binding mitochondrial carrier protein Aralar2
	SLC39A6	Solute carrier family 39 (zinc transporter)
	ANKRD32	Ankyrin repeat domain 32
miR-495	RLF	Zinc finger protein Rlf (Rearranged L-myc fusion gene protein)
	ARHE	Rho-related GTP-binding protein RhoE (Rho8)
	CTNND2	Catenin delta-2 (delta-catenin)
	NP_620711	Delta-notch-like EGF repeat-containing transmembrane
	NEIL2	Nei-like 2 (DNA glycosylases)
	FGF14	Fibroblast growth factor-14
miR-200b	PERP	TP53 apoptosis effector
	BAG2	BCL2-associated athanogene 2
	ZFHX1B	Zinc finger homeobox protein 1b
miR-224	CDKN2D	Cyclin-dependent kinase 4 inhibitor D (p19-INK4d)
	CDKN1A	Cyclin-dependent kinase inhibitor 1 (p21)
miR-100	HOXA1	Homeobox protein Hox-A1
	TNFSF6	Tumour necrosis factor ligand superfamily member 6
	UST	Uronyl-2-sulphotransferase
miR-99a	HOXA1	Homeobox protein Hox-A1
	UST	Uronyl-2-sulphotransferase

increase in mRNA and protein level of MRP1/ABCC1 by miR-134 antagomir treatment (Figs. 5 and 6).

4. Discussion

Chemotherapy remains as the essential component for the treatment of SCLC patients. Despite a good initial response to therapy, most SCLC patients suffer from the development of chemotherapy resistance and relapse. Therefore, it is urgently required to develop novel targeting specific molecules involved in drug resistance and establish rational therapeutic approaches. Microarray analysis has made it possible to identify the appropriate candidate molecules for such purposes. Cellular models of SCLC *in vitro* are based on assays using sensitive and resistant cell lines to chemotherapy to mimic the clinical scenario. NCI-H69 (sensitive) and NCI-H69AR (anthracycline-resistant) cell lines, purchased from ATCC (American Type Culture Collection), have been characterised in the multidrug resistance.[26–28] In this study, we firstly reported the comprehensive miRNA and mRNA expression profiles of SCLC involved in drug resistance by miRNA microarray and cDNA microarray.

Recently, the evidence of the roles for miRNAs in determining drug sensitivity/resistance has been emerging.

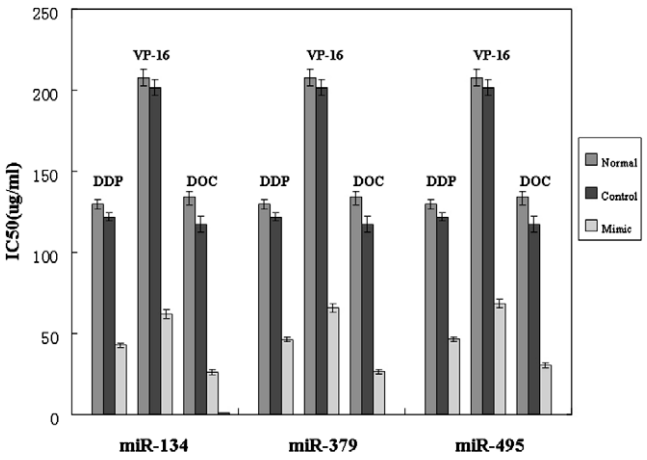

Fig. 1 – Influence of miRNA mimics on cell susceptibility to chemotherapeutic drugs. Following transfection of the H69AR cells with the mimics of miR-134, miR-379 and miR-495, respectively, the sensitivity to Cisplatin (DDP), Etoposide (VP-16) and Doxorubicin (DOX) was significantly increased by 2.79–5.17-fold in H69AR cells.

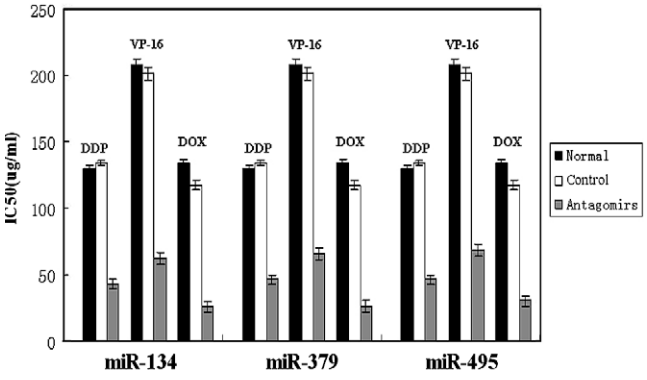

Fig. 2 – Influence of miRNA antagomirs on cell susceptibility to chemotherapeutic drugs. Following transfection of the H69AR cells with the antagomirs of miR-134, miR-379 and miR-495, respectively, the growth inhibition by Cisplatin (DDP), Etoposide (VP-16) and Doxorubicin (DOX) in H69AR cells was 1.37–1.53-fold greater in the antagomir-treated cells than that in control cells.

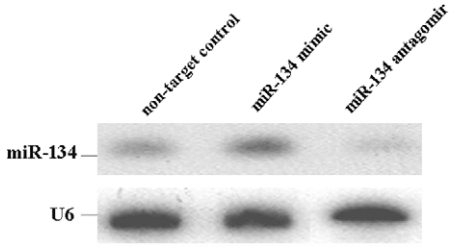

Fig. 3 – Northern blot showing up-regulation of miR-134 following transfection of the H69AR cells with miR-134 mimic, while down-regulation in the cells with miR-134 antagomir as compared with the control. U6 small RNA was used as the control.

A panel of six miRNAs (let-7e, miR-30c, miR-125b, miR-130a and miR-335) was always diversely expressed in drug-resistant ovarian cancer cells. These miRNAs could be involved in the regulation of genes involved in Cisplatin and Paclitaxel P-glycoprotein-independent cross-resistance.[34] The expressions of miR-27a, miR-99a, miR-100, miR-125b1 and miR-451 were increased by 2.0–7.8-fold in human MDR ovarian cancer cell line A2780DX5 and cervix carcinoma cell line KBV1, as compared to their respective parental cell lines.[19] Aberrant miRNA expression also showed significant (>1.8-fold) up-regulation of eight miRNAs and marked down-regulation (>50%) of seven miRNAs in a tamoxifen-resistant breast cancer cell line compared to the tamoxifen-sensitive cell line.[35] In the present report, we compared the profiling of miRNA expression and showed a differential expression pattern between H69AR cells and its parental drug sensitive cells. Of the 852 miRNA genes analysed, 61 miRNAs are presented significantly (>3-fold) including up-regulation of 24 miRNAs and down-regulation of 37 miRNAs. Among these miRNAs, 48 of 61 differentially expressed miRNAs were firstly reported to be closely associated with drug resistance excluding 13 miRNAs (miRNA-200b/c, miRNA-335, miRNA-145, miRNA-214, miRNA-99, etc.) which were overexpressed in various types of cancer cells.[17–20] The up-regulation and down-regulation of these differentially expressed miRNAs in the H69AR cells suggest a role for these small RNA molecules in the development of drug resistance in SCLC cells.

Many previous studies have showed that some of the known miRNAs appear in clusters on a single polycistronic transcript. The fraction of clustered miRNA genes was estimated to be around 50% in *D. melanogaster*[36] and 37–45.45% in human.[37–40] In this study, 37.7% (24/61) of miRNA genes were organised as 10 clusters in total 61 significantly expressed miRNAs. The miR-134 cluster contains a huge cluster of 13 miRNA genes within 10 kb on chromosome 14q32.31. There are two miRNA genes in other nine clusters. It has been reported that miR-134 cluster is identified to be the largest miRNA cluster to date and is comprised of greater than 50 members.[41] Therefore, the miR-134 cluster represents an extremely large polycistronic transcript possibly regulated by differential methylation in a region which is a frequent target of translocations and inversions in T cell leukaemia.[42] Our results suggest that the expression of miRNAs in a cluster may be co-regulated, and that they may play a common molecular role in drug resistance of SCLC.

A range of algorithms including TargetScan, miRBase and PicTar has been developed to predict the genes targeted by specific miRNAs. These algorithms suggested that conserved human miRNAs target at least 30% of all human genes and that each miRNA targets the potential mRNAs from hundreds to thousands.[43–45] It becomes possible to obtain lists of the potential target mRNAs for each miRNA. However, only a small number of predicted target genes regulated by miRNAs have been experimentally verified. So we next asked whether there is an association between the expression pattern of a miRNA and that of a known target in drug-resistant SCLC cells. With the discovery of specific miRNAs and its targets for drug-resistant SCLC cells, we calculated the correlation between the miRNA and mRNA expression profiles obtained above by MAS software, as well as by querying the PubMed

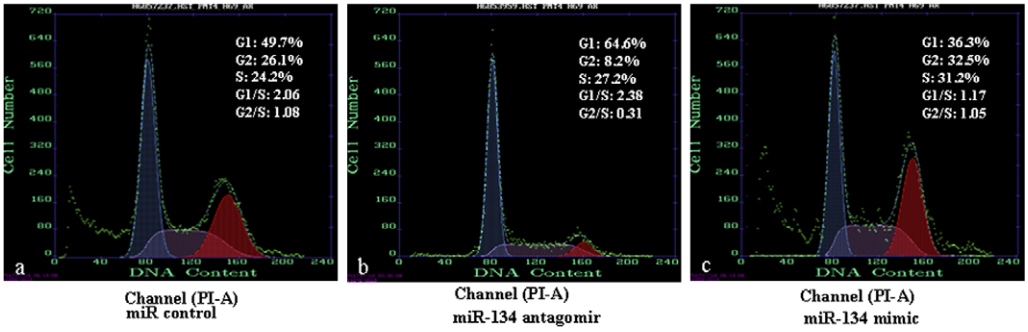

Fig. 4 – Impact of miR-134 on cell cycle control. Cell cycle analysis by flow cytometry in H69AR cells transfected with miR-134 antagomir (b), mimic (c) and the control (a), respectively.

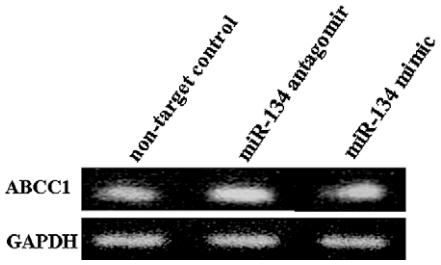

Fig. 5 – RT-PCR showing MRP1/ABCC1 expression in mRNA level in miR-134 mimic-transfected cells and miR-134 antagomir-transfected cells.

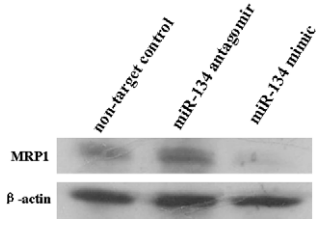

Fig. 6 – Western blot showing MRP1/ABCC1 protein expression in miR-134 mimic-transfected cells and miR-134 antagomir-transfected cells.

database. After detecting a signal we subsequently tested alternative miRNA target gene prediction algorithms. For every mRNA expression dataset, the mRNA expression of predicted targets was mapped on the respective miRNA families. We found that only 27 of 69 miRNAs were significantly correlated with 604 of 21,522 70 mRNA transcripts. Forty-one mRNAs may be the putative targets of miR-134, miR-379, miR-382, miR-494 and miR-495 in miR-134-cluster. For example, analyses indicated that miR-134 may mediate changes in the ARHGDIB expression which is a substrate of caspase-3 and closely associated with apoptosis. miR-379 is likely to be involved in transcriptional regulation of TADA2L and MAP4K5 in the assembly of transactivation-competent beta-catenin complexes at Wnt target genes or in MAPK pathways. Because miRNAs are predicted to target multiple unrelated genes that are not coexpressed,[46,47] it is not surprising that only a few of miRNAs tend to be strongly correlated with the particular target transcripts.

To further determine whether these differentially expressed miRNAs could modulate the sensitivity of H69AR cells to chemotherapeutic drugs, mimics and antagomirs of miR-134 cluster including miR-134, miR-379 and miR-495 were selectively tested in H69AR and H69 cells. Following transfection of H69AR cells with the antagomirs or mimics of miRNAs as stated above, we treated the cells with chemotherapeutic drugs Cisplatin, Etoposide and Doxorubicin. The results showed that treatment with the mimics of miR-134, miR-379 and miR-495 greatly increased the sensitivity of H69AR cells to chemotherapeutic drugs. Meanwhile, sensitivity to these drugs was reduced in treatment with the antagomirs of miR-134, miR-379 and miR-495. Thus, our findings suggest that miR-134 cluster could play an important role in chemoresistance of SCLC. One reason of the resistant phenotype in H69AR cells may be that miR-134 increases the cell survival by inducing G1 arrest.

It has been well documented that MRP1/ABCC1 contributes to chemoresistance in H69AR.[26–28] Therefore, it is necessary to investigate whether miR-134 is involved in the regulation of MRP1/ABCC1 expression. In the present study, we showed that MRP1/ABCC1 is negatively regulated by miR-134 and that down-regulation of MRP1/ABCC1 at the protein level largely correlates with elevated levels of miR-134 in H69AR cells. These data indicate that miR-134 could be a causal factor of the down-regulation of MRP1/ABCC1 in H69AR cells.

In summary, our observation demonstrated that specific miRNAs are regulated in drug-resistant SCLC cells, thereby suggesting their involvement in the generation of the drug-resistant phenotype, as directly demonstrated by the case of miR-134, which targets the expression of MRP1/ABCC1. The findings indicate the potential utility of these miRNAs as specific diagnostic biomarkers for drug sensitivity of SCLC. These miRNA signatures need to be further validated in clinical samples from SCLC patients. In addition, targeting these differentially expressed miRNAs may offer novel therapeutic approaches for the treatment of SCLC.

Conflict of interest statement

None declared.

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