Journal of Cellular and Molecular Medicine
A JOURNAL OF TRANSLATIONAL MEDICINE

J. Cell. Mol. Med. Vol 15, No 10, 2011 pp. 2164-2175

Down-regulated miR-331–5p and miR-27a are associated with chemotherapy resistance and relapse in leukaemia

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Received: April 21, 2010; Accepted: November 2, 2010

Abstract

Multidrug resistance (MDR) and disease relapse are challenging clinical problems in the treatment of leukaemia. Relapsed disease is frequently refractory to chemotherapy and exhibits multiple drug resistance. Therefore, it is important to identify the mechanism by which cancer cells develop resistance. In this study, we used microRNA (miRNA) microarray and qRT-PCR approaches to investigate the expression of miRNAs in three leukaemia cell lines with different degrees of resistance to doxorubicin (DOX) compared with their parent cell line, K562. The expression of miR-331–5p and miR-27a was inversely correlated with the expression of a drug-resistant factor, P-glycoprotein (P-gp), in leukaemia cell lines with gradually increasing resistance. The development of drug resistance is regulated by the expression of the P-gp. Transfection of the K562 and, a human promyelocytic cell line (HL) HL60 DOX-resistant cells with miR-331–5p and miR-27a, separately or in combination, resulted in the increased sensitivity of cells to DOX, suggesting that correction of altered expression of miRNAs may be used for therapeutic strategies to overcome leukaemia cell resistance. Importantly, miR-331–5p and miR-27a were also expressed at lower levels in a panel of relapse patients compared with primary patients at diagnosis, further illustrating that leukaemia relapse might be a consequence of deregulation of miR-331–5p and miR-27a.

Keywords: leukaemia • drug resistance • relapse • miR-331–5p • miR-27a • P-glycoprotein

Introduction

Leukaemia is one of the leading causes of cancer death worldwide. Although the current chemotherapy has remarkably improved the cure rate over the past decade, a number of patients still die of the disease. Drug resistance remains the major clinical obstacle to successful treatment of leukaemia patients and leads to poor prognosis compared to patients with relatively sensitive cells at the initial diagnosis [1]. Several studies have revealed that causes of cancer-specific drug resistance are linked to random drug-induced mutational events (genetic hypothesis), drug-induced non-mutational alterations of gene function (epigenetic hypothesis) and drug-induced karyotypic changes (karyotypic hypothesis) [2–5]. Some evidence suggests that the multiple drug resistance (MDR) protein 1 gene (*MDR1*) is activated during emerging resistance to anti-neoplastic drugs, resulting in overexpression of P-glycoprotein (P-gp), thus confers cancer cell resistance to a broad range of structurally and functionally diverse chemotherapeutic drugs, including drugs against leukaemia [6–8]. However, the detailed mechanism of the development of resistance in cancer cells is still not fully understood.

Recently, microRNAs (miRNA), a class of non-coding RNA, were found to play important roles in various fundamental biological processes, such as cell proliferation, apoptosis and differentiation. Some miRNAs are involved in cancer regulation and are considered as oncogenes or tumour suppressors, thus representing

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doi:10.1111/j.1582-4934.2010.01213.x

promising diagnostic and prognostic markers in drug resistance/sensitivity [9–11]. Yang *et al*. reported that miR-214 negatively regulated phosphatase and tensin homolog (PTEN), which is a tumour suppressor, leading to induced cell survival and cisplatin resistance [12]. Xia *et al*. also showed that miR-15b and miR-16 modulated MDR by targeting BCL2 in human gastric cancer cells [13]. More recently, miR-451 and miR-326 were found to be inversely correlated with *MDR1* and MDR-associated protein 1 (*MRP1*) expression in drug-resistant breast cancer cells [14, 15]. It was also reported that up-regulated miR-27a and miR-451 could stimulate *MDR1* expression in cell lines of human ovarian cancer and cervix carcinoma [16]. However, the role of miRNAs in drug resistance and clinical relapse samples refractory to chemotherapy in leukaemia has not been fully addressed yet.

Here, we report for the first time that down-regulated miR-331–5p and miR-27a are associated with doxorubicin (DOX) resistance in leukaemia K562 cell lines. Overexpression of miR-331–5p and/or miR-27a can effectively increase the drug sensitivity of K562 and HL60 DOX-resistant cells. Furthermore, we demonstrate that the drug-resistance mechanism mediated by miR-331–5p and miR-27a and confirmed their expressions in clinically untreated, complete remission (CR) and relapsed patients of leukaemia. This study might provide a better understanding of a new mechanism of miRNAs-mediated drug resistance in leukaemia.

Materials and methods

Cell culture and DOX-resistant cell selection

Three DOX-resistant K562 cell lines (K562/DOX), K562/DOX1, K562/DOX2 and K562/DOX3, were selected from parental K562 cells [K562/wide type (WT)] by prolonged treatment with 0.1, 0.5 and 1 μg/ml DOX, respectively, as described by Davies *et al.* [17]. Selected cells were washed free of DOX prior to the experiment. K562/WT and the selected K562/DOX cells were cultured using RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% foetal bovine serum (Thermo Scientific HyClone, Logan, UT, USA) at 37°C in a 5% CO_2 atmosphere. Two other myeloid leukemic cell lines NB4, a human promyelocytic leukaemia cell line, and HL60, as well as their drug-resistant cells HL60/DOX (subclone of HL60 with DOX resistance established by drug inducing progressively) and NB4/R2 [subclone of NB4 with all-trans retinoic acid (ATRA)-resistance established by mutating the E domain of PML/RARα, which is a chimeric gene of promyelocytic leukemia retinoic acid receptor α] were also applied for comparison in this study.

Patients' samples

In total, 48 clinical samples were collected from patients enrolled in this study, namely 26 lymphocytic leukaemia and myeloid leukaemia samples at primary diagnosis, 22 samples after therapy from the First and Second Affiliated Hospital of Sun Yat-sen University and Beijing Children's Hospital. Patients' characteristics were available for all of the patients (see Table S1). Bone marrow was collected from patients by bone marrow puncture at diagnosis or at follow-up after therapy. Written informed consent for the biological studies was obtained from the parents/guardians. The study was approved by the ethics committee of the affiliated hospitals of the Sun Yat-sen University.

Immunofluorescent staining

Expression of P-gp protein in the K562/WT and K562/DOX cells was detected by immunofluorescence staining, as described previously [18]. Cells were collected and washed twice in PBS. The washed cells were smeared on a glass cover-slip and fixed in PBS containing 4% paraformaldehyde. The fixed cells were incubated with a primary mouse anti-human P-gp monoclonal antibody (Merck Biochemicals, Darmstadt, Germany), diluted 1:50 at 4°C overnight. A rhodamine-coupled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used for P-gp visualization at 1:200 dilution and 1 mM Hoechst (Invitrogen) was used for counterstaining of the nuclei at 1:500; the secondary staining was performed at room temperature for 30 min.

Flow cytometry

P-gp surface expression was analysed by flow cytometry (Beckman Coulter, Brea, CA, USA) using the fluorescent-labelled monoclonal antibody, UIC, a mouse monoclonal antibody against the extracellular conformational epitope of P-glycoprotein (Pgp) (CD243)-Phycoerythrin (PE), a fluorescence marker (Beckman Coulter). A total of 1×10^6 K562/WT, HL60/WT, NB4, K562/DOX, HL60/DOX and NB4/R2 cells were washed twice in PBS and incubated for 1 hr at 4°C. Then, the PE-conjugated anti-mouse antibodies IgG2a and P-gp (CD243) were added to the corresponding sensitive and resistant cells. After 30 min. of incubation at room temperature, cells were washed twice with PBS and resuspended in 300 μl of PBS. Propidium iodide was added just before analysis to exclude dead cells from the fluorescence measurement. An isotypic control was measured in parallel to determine non-specific binding.

RNA extraction and miRNA microarray analysis

Total RNA was extracted from K562/WT and K562/DOX cells with Trizol (Invitrogen), according to the manufacturer's instructions. MiRNA microarray analysis was performed with an miRNA microarray chip (Capital Bio, Beijing, China) containing 743 probes in triplicate, corresponding to 576 human (including 122 predicted miRNAs sequences from published reference [19]), 238 rat and 358 mouse mature miRNAs found in the miRNA Registry (http://microrna.sanger.ac.uk/sequences/; miRBase14; September 2009). Processing of each sample was repeated three times.

The miRNA detection signal threshold was defined as twice the maximum background signal. The maximum signal level of the background probes was 180. Normalization was done using a cyclic LOWESS (locally weighted regression) method to remove the system-related variations. The data adjustments included data filtering, log2 transformation and gene centring and normalization. K562/WT and K562/DOXs samples were compared using t-tests, and miRNA with *P*-values <0.05 were selected as significant for cluster analysis. The cluster analysis was done using a hierarchical method, average linkage and Euclidean distance metrics [20].

Quantitative real-time PCR analysis for miRNA expression

Quantitative real-time PCR (qRT-PCR) was performed to detect mature miRNAs. Briefly, 0.2 μg of small RNA extracted from cell samples was reverse-transcribed to cDNA using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA) and amplified with specific designed miRNA RT-primers and PCR amplification primers (Sangon, Shanghai, China). Sequences of all the primers are shown in Table S2. The expression level of each miRNA was measured using the $2^{-\mathrm{DeltaDeltaCt}}$ method [21]. The results were presented as the fold change of each miRNA in the drug-resistant cells relative to the parental sensitive cells.

Western immunoblotting

Total cellular extracts were prepared by homogenization of 3×10^6 to 5×10^6 cells in 100–300 μl radioimmunoprecipitation assay lysis buffer containing 1:1000 phenylmethanesulfonyl fluoride (PMSF); (Beyotime, Guangzhou, China). Lysates were incubated at 4°C for 30 min. followed by centrifugation at 13,000 rpm at 4°C for 30 min. Equal amounts of proteins (2 μg) and 10 μl loading control (Fermentas, Shenzhen, China) were separated by 8% SDS-PAGE and transferred to polyvinylidene difluoride membranes (GE Healthcare Biosciences, Pittsburgh, PA, USA). The membranes were incubated with antibodies against P-gp (0.5 μg/ml; Merk Biochemicals, Darmstadt, Germany). Antibody binding was assessed by incubation with horseradish peroxidase-conjugated antimouse secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Chemiluminescence was detected using an ECL Plus immunoblotting detection system (GE Healthcare Biosciences).

Cell transfection

The miR-331–5p and miR-27a mimics were purchased from GenePharm (Shanghai, China). The three items of si-h-MDR1 were purchased from RiboBio (GuangZhou, China). Mimics or scrambled oligonucleotides (negative control, NC) were transfected into K562/DOX1, HL60/DOX and NB4/R2 cells at a final concentration of 100 nM, or 50 nM of miR-331–5p or miR-27a when co-transfected, and siRNAs (100 nM) were individually or combined transfected into K562/DOX1 cells using Lipofectamine 2000 (Invitrogen). The transfected cells were collected to exact RNA used for qRT-PCR or measure the protein levels of P-gp at 48 hrs after transfection.

Construction of the vector and luciferase reporter assay

The synthesized 59 bp 3′-UTR (Sangon) of MDR1, containing the miRNA response element (MRE) or deletion mutants of the MRE for miR-27a or miR-331–5p, were annealed and ligated into the psiCheck-2 vector (Promega) immediately 3′ downstream of the Renilla luciferase gene to obtain the MDR1 wild-type (psi-MDR1) and mutants (psi-MDR1-M). The synthesized top (sense) and bottom (antisense) strands of each MRE were designed to contain XhoI and NotI sites, respectively. The MDR1 wild-type and mutant insertions were confirmed by DNA sequencing. All primer information is available in Table S2.

A total of 5×10^4 HEK-293T cells were grown in DMEM containing 10% Foetal Bovine Serum (FBS) in 48-well plates at 37°C in a humidified atmosphere of 5% CO_2. The cells were transfected using Lipofectamine 2000 with *Renilla luciferase* and *firefly luciferase* reporter constructs and miRNA mimics (50 nM). *Firefly* and *Renilla luciferase* activities were measured immediately using dual-luciferase assays (Promega) 24 hrs after transfection, according to the manufacturer's instructions.

Measurement of drug sensitivity by the MTT assay

A total of 3 × 105 K562/DOX1, HL60/DOX or NB4/R2 cells were transfected with miR-27a, miR-331–5p, both miRNAs or the scrambled oligonucleotides in a 24-well dish. The cells were collected and seeded in 96-well plates 48 hrs after transfection. Fresh medium containing DOX at concentrations ranging from 0.01 to 500 μg/ml was added immediately. The cells were incubated for 48 hrs at 37°C. The surviving cells were estimated using the MTT assay and the IC50 was calculated. The experiments were repeated twice, and each cell line was tested in triplicate.

Statistical analysis

Correlations were determined using Pearson correlation coefficient (*r*), and a Fisher *r*-to-*z* transformation was carried out to calculate a probability level (*P*-value). Student's t-test was performed to assay the statistical significance.

Results

Expression of P-gp in K562/WT and K562/DOX leukaemia cell lines

Because increased expression of P-gp is involved in cancer cell resistance to chemotherapeutic agents [22], we first analysed the expression of P-gp in K562/WT and three DOX-resistant cell lines, K562/DOX1, K562/DOX2 and K562/DOX3, by immunofluorescence staining (Fig. 1A) and Western blotting (Fig. 1B). P-gp proteins were overexpressed in the three DOX-resistant cell lines compared to K652/WT cells. Furthermore, the expression of P-gp was related to the DOX resistance of the cells; greater DOX resistance was linked to higher P-gp expression. These results were corroborated by the quantification of P-gp expression in the three DOX-resistant K562 cell lines by FACS (Fig. 1C). We also detected the expression of P-gp in HL60, NB4 and their drug-resistant cells, HL60/DOX and NB4/R2, and found that the expression of P-gp was not so strongly in HL60/DOX compared with K562/DOX1, which is consistent with the report from Baran *et al.* [23], while, expectedly, there is no P-gp expression in NB4/R2 (Fig. 3A and B).

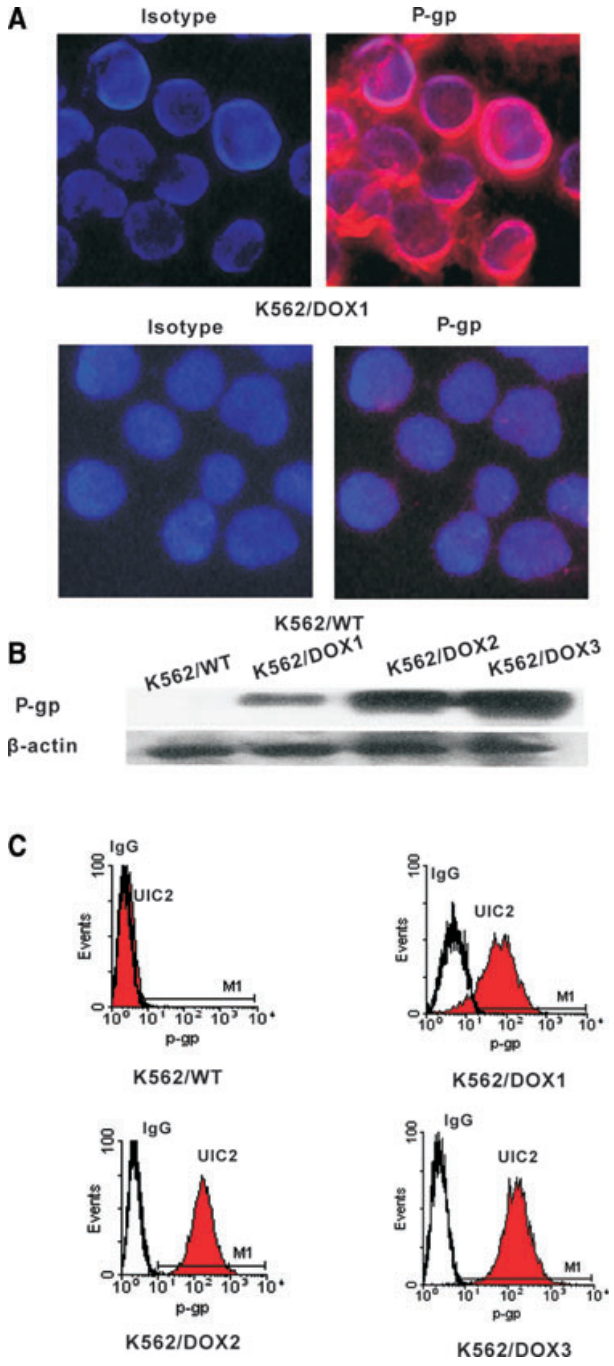


Fig. 1 Expression of P-gp in K562/WT and K562/DOXs cells. (**A**) Immunofluorescence staining for P-gp in K562/WT and K562/DOX1cells. Red colour represents expression of P-gp and blue colour is the nuclear counter-stain. (**B**) Expression of P-gp gradually increases with the increased concentrations of DOX. K562/DOX1, K562/DOX2 and K562/DOX3. DOX-resistant cells were selected by prolonged treatment with 0.1 μg/ml, 0.5 μg /ml and 1 μg/ml DOX, respectively. (**C**) Flow cytometry analysis of P-gp surface expression using the UIC2-phycoerythrin-conjugated antibody (filled red peak) and the corresponding IgG isotype control (clear peak).

MiRNA expression profile in K562/WT and K562/DOXs cells

To identify deregulated miRNAs associated with DOX resistance in leukaemia cell lines, we determined the miRNA expression profile of K562/WT and three K562/DOX cell lines using an miRNA microarray. Figure 2A summarizes the deregulated miRNAs in all three DOX-resistant cell lines, including data evaluated using cluster analysis. To confirm the differential expression pattern, we performed qRT-PCR for miR-20a, miR-27a, miR-188–3p miR-214, miR-223, miR-331–5p, miR-338–5p, miR-15a and miR-455–3p in K562/WT and DOX-resistant cells. As shown in Figure 2B–E, the differential regulation of these miRNAs was confirmed by qRT-PCR, except for miR-223, whose expression pattern was opposite to that in the microarray data.

To further elucidate whether the DOX resistance of the cells affects the miRNA expression patterns, we measured the expression levels of the selected miRNAs (miR-15a, miR-27a, miR-455–3p, miR-200a, miR-338–5p and miR-331–5p) by qRT-PCR in three DOX-resistant cell lines, K562/DOX1, K562/DOX2 and K562/DOX3. We found that the expression levels of two miRNAs, miR-331–5p and miR-27a, were highest in the K562/WT cells, while decreased in the three DOX-resistant cell lines (Fig. 2D and E), suggesting that the expression patterns of these two miRNAs were inversely correlated with the expression of P-gp in the three K562/DOX cell lines. We also detected the two miRNAs expression in HL60 and NB4 and their drug-resistant cells, HL60/DOX and NB4/R2, respectively. We found that miR-331–5p and miR-27a were down-regulated in the HL60/DOX cells that exist P-gp even though the P-gp expression level is low in the cells. However, for NB4/R2, which is P-gp-dissociated ATRA-resistant cells and has no expression of P-gp, there was no obvious regularity of the two miRNAs expression (Fig. 3C and D). These results suggest that miR-331–5p and miR-27a might be involved in drug resistance. As both two miRNA showed a clearly inverse correlation with P-gp expression in K562 cells, as well as a weak correlation in HL60 cells, we therefore, focused on these two deregulated miRNAs in the following studies.

MiR-331–5p and miR-27a regulate translation of MDR1

Because P-gp expression was inversely correlated with miR-331–5p or miR-27a levels in K562/DOXs and HL60/DOX, we suggested that miR-331–5p and miR-27a might specifically and directly regulate the translation of *MDR1*. To test our hypothesis, the potential miRNA binding sites in the 3′-UTR of the *MDR1* gene were analysed using a bioinformatics strategy. Searches using TargetScan [24] and miRBase [25] yielded more than 30 miRNAs, including miR-331–5p and miR-27a (Fig. 4A, upper panel). Based on the bioinformatics analysis, we constructed a recombinant luciferase reporter system that combined a 3′-UTR segment of *MDR1* with the putative miR-331–5p

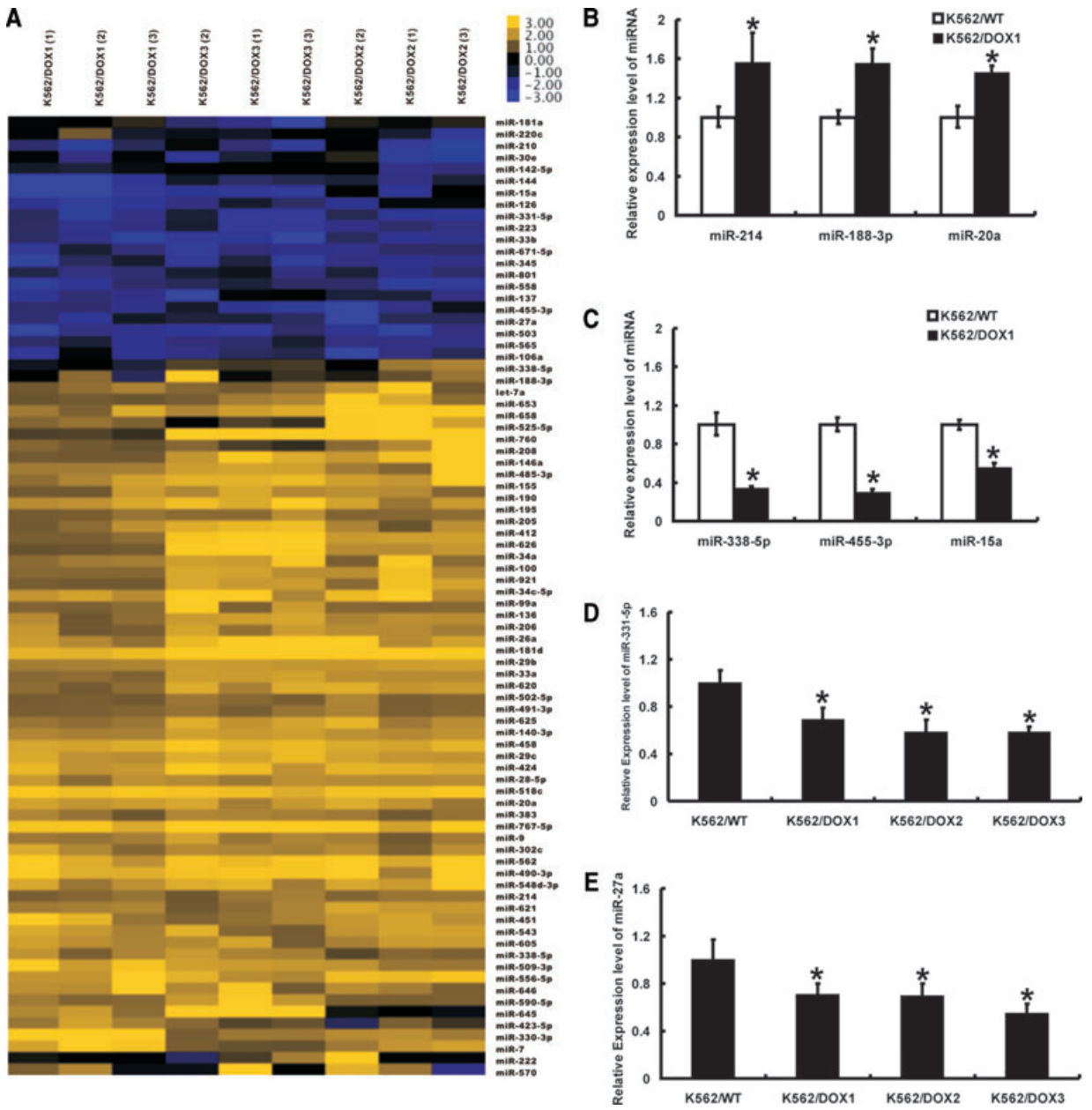


Fig. 2 miRNA cluster analysis and qRT-PCR confirmation. (**A**) The top 83 differentially expressed miRNAs in the DOX-resistant cells. (1), (2) and (3) indicate three repeat array chips from each DOX-resistant cell line. Right upper panel: colour bar indicates fold changes of DOX-resistant cells *versus* K562/WT cells. The average miRNA expression of K562/WT cells was set as 1. (**B**, **C**) Selected miRNA expression was validated with qRT-PCR. (**D**, **E**) Expression of miR-27a and miR-331–5p were gradually down-regulated in the three DOX-resistant K562 cell lines compared to the sensitive cells. Error bars represent standard deviation and were obtained from three independent experiments $^{*}P < 0.05$, compared with K562/WT cells.

and miR-27a binding sites, psi-MDR1. Moreover, using the same system we made a psi-MDR1-M construct containing an miR-MRE with the miR-331–5p and miR-27a binding sites deleted (Fig. 4A, lower panel). The psi-MDR1 or psi-MDR1-M was co-transfected into cultured HEK-293T cells with miR-331–5p or miR-27a mimics.

As shown in Figure 4B, the transfection of miR-331–5p resulted in about 40% reduction of luciferase activity in the psi-MDR1-

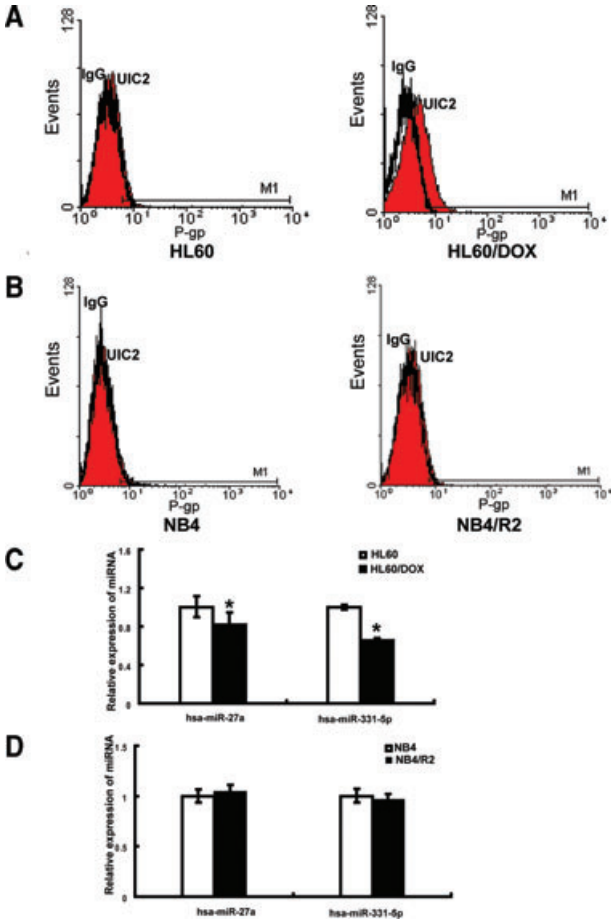


Fig. 3 Expression of P-gp, miR-27a and miR-331–5p in sensitive and drug–resistant HL60 and NB4 cells.(**A**) Flow cytometry analysis of P-gp expression in HL60 and HL60/DOX cells. (**B**) Flow cytometry analysis of P-gp expression in NB4 and NB4/R2 cells. (**C**) Expression of miR-27a and miR-331–5p in HL60 and HL60/DOX cells using qRT-PCR. $^{*}P < 0.05$, compared with HL60 cells. (**D**) Expression of miR-27a and miR-331–5p in NB4 and NB4/R2 cells using qRT-PCR. $P > 0.05$, compared with NB4 cells. Error bars represent standard deviation.

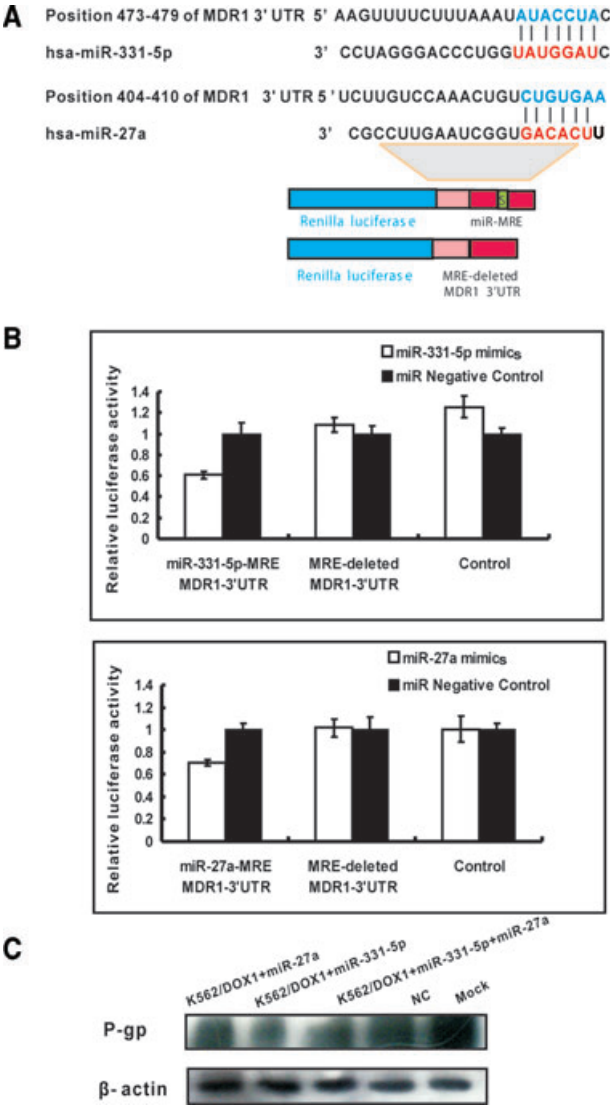


Fig. 4 Elevated levels of miR-27a and miR-331–5p, individually or in combination, inhibit expression of P-gp proteins in K562/DOX cells. (**A**) The putative miR-27a and miR-331–5p target sequence in the *MDR1* gene. (**B**) Luciferase reporter assay in 293T cells. Expression of the *MDR1* 3′-UTR fragment, with the binding sequence for miR-27a and miR-331–5p, was inhibited by transfection of miR-27a (50 nM) and miR-331–5p (50 nM), respectively. The deleted region included the binding sites for miR-27a and miR-331–5p. (**C**) Protein expression levels of MDR1 in K562/DOX1 cells transfected with miR-27a and miR-331–5p, individually or in combination, were down-regulated compared to K562/DOX1 cells transfected with the scrambled oligonucleotides (NC). Error bars represent standard deviation. Three independent experiments were performed.

transfected HEK-293T cells. However, it did not affect reporter activity in the psi-MDR1-M-transfected HEK-293T cells. The luciferase activities showed almost no change in wild-type and mutant *MDR1* reporter-transfected HEK-293T cells when miR-331–5p was deleted. Similar results were obtained when HEK-293T cells were transfected with miR-27a (Fig. 4B). These results suggest that *MDR1* is a target of miR-331–5p and miR-27a and that the putative miR-331–5p and miR-27a binding sites are critical for regulating the expression of MDR1 by miR-331–5p and miR-27a.

To elucidate whether miR-331–5p and miR-27a affect the protein levels of P-gp in K562 DOX-resistant cells, we transfected K562/DOX1 cells with miR-331–5p or/and miR-27a. P-gp expression was determined by Western blotting at 48 hrs after transfection. Figure 4C shows that transfection of K562-resistant cells with miR-27a or miR-331–5p, or a combination of miR-27a and miR-331–5p, resulted in a decrease in P-gp levels. However, the combination of these two miRNAs had more inhibitory activity than any single miRNA.

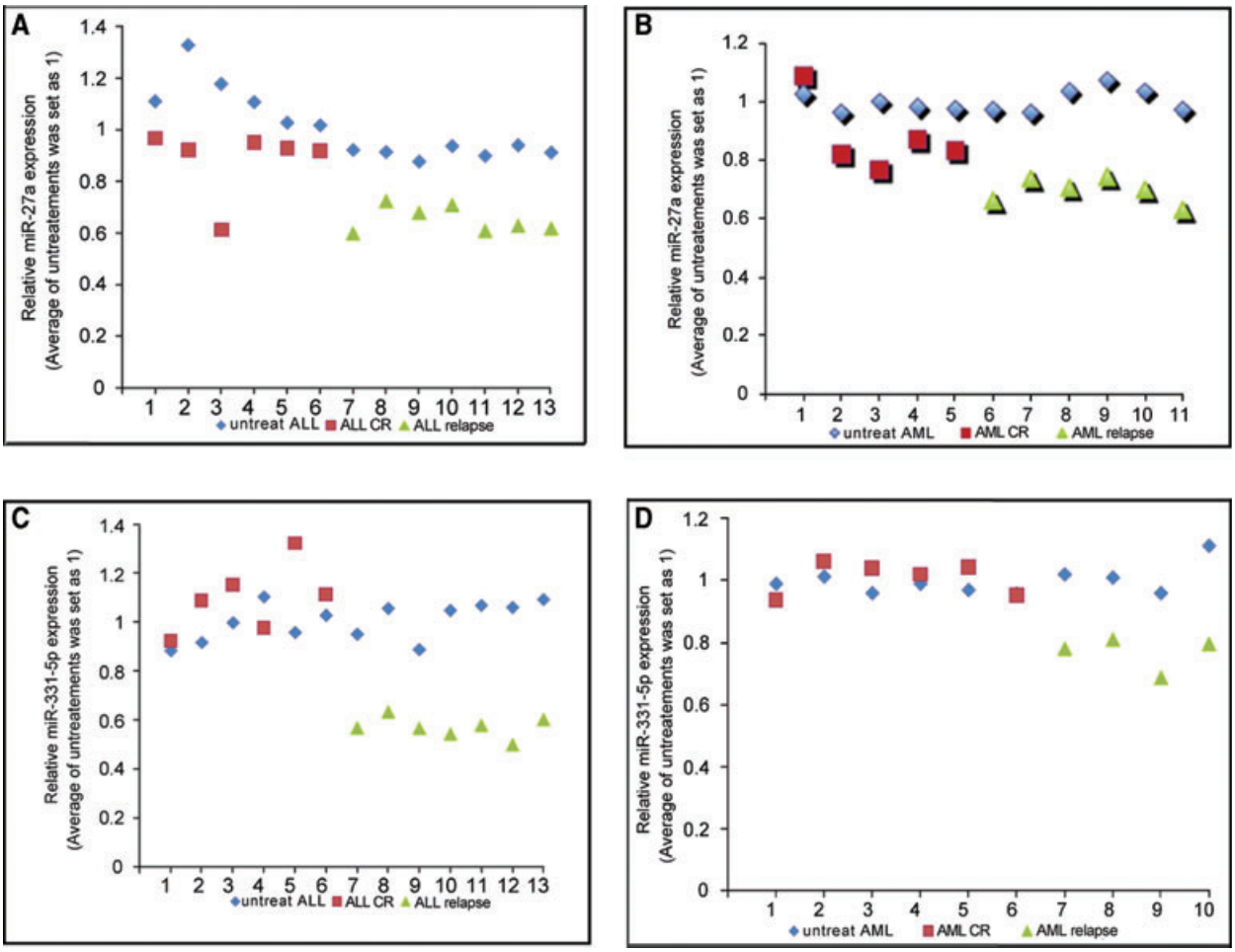


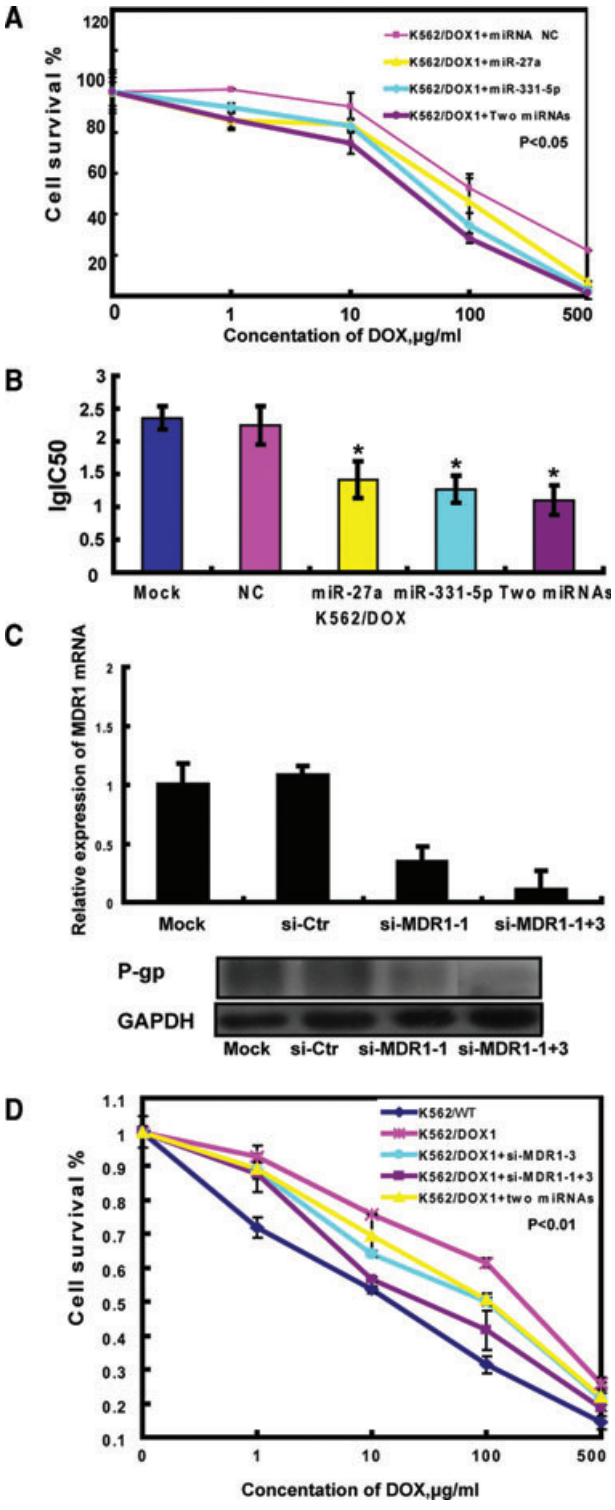
Fig. 5 Different expression of miRNAs in untreated patients, CR patients and relapsed patients. miRNAs were detected with real-time qRT-PCR. The data were normalized against U6 expression in the same patient. The average levels of miRNAs in patients at diagnosis were set as 1 in all of the subfigures, and the levels of miRNAs in patients were expressed as the fold changes against those in patients at diagnosis. (**A**, **B**) Relative miR-27a expression in ALL and AML patients ($P < 0.01$). (**C**, **D**) Relative miR-331–5p expression in ALL and AML patients ($P < 0.01$). The miRNA data of the same patient, before and after treatment, are listed in same column. All *P*-values indicate average miRNA expression of relapsed patients is statistically compared with untreated patients.

MiR-331–5p and miR-27a are associated with relapse risk

It has been reported that P-gp expression is higher at relapse compared with the initial leukaemia or is related to the long-term survival or relapse risk [7]. The correlation of miR-331–5p and miR-27a with P-gp expression in DOX-resistant leukaemia cells led us to investigate whether these two miRNAs were also related to the relapse risk. Therefore, we analysed the expression of these two miRNAs in 48 clinical leukaemia samples, including 26 patients at primary diagnosis, 11 with CR after treatment and 11 that relapsed with 3-year clinical follow-up. The patients' profiles are summarized in Table S1. Figure 5 shows the expression profiles of miR-331–5p and miR-27a in paired lymphocytic leukaemia and myeloid leukaemia patients before and after therapy. The expression of both miRNAs in lymphocytic leukaemia and myeloid leukaemia patients with relapse were lower than in patients at diagnosis as well as in CR patients. These results suggest that down-regulated miR-331–5p and miR-27a might be involved in the process of leukaemia relapse.

MiR-331–5p and miR-27a can increase the sensitivity of K562/DOX and HL60/DOX cells to DOX

Reduced miR-331–5p and miR-27a expression in the drug-resistant leukaemia cells and in the clinical samples from the



relapse patients suggested that down-regulation of these two miRNAs contributes to anti-cancer drug resistance. We suggested that the restoration of these two miRNAs in K562/DOX cells might increase their sensitivity to DOX. MTT assay showed that transfection with either miR-331–5p or miR-27a could reduce the proliferation rate of K562/DOX1, indicating increased sensitivity to DOX treatment (Fig. 6A). The IC50 of DOX treatment in K562/DOX1 cells transfected with miR-331–5p or miR-27a was lower than in the same cells transfected with scrambled oligonucleotides ($P < 0.05$) (Fig. 6B). Co-transfection of miR-331–5p and miR-27a significantly enhanced the sensitivity to DOX treatment in DOX-resistant leukaemia cells. The same result was found in HL60/DOX cells, whereas no effect was observed for the NB4/R2 cells which are resistant to ATRA (Fig. 7A–C). This indicates a synergistic regulation mechanism of miR-331–5p and miR-27a in drug resistance.

To further confirm that P-gp down-regulation is important for reversing the drug-resistance. RNAi against MDR1 was performed to analyse its role in drug sensitivity assay. The transfection of K562/DOX1 cells with different items of siRNAs against the MDR1 mRNA resulted in a strong decrease in the levels of the endogenous protein P-gp, especially for the two items of si-MDR1–1 and si-MDR1–3 (Fig. 6C). Also, the results demonstrated that cell survival curves by overexpressing the miRNAs or by down-regulating MDR1 were closer to the parental cells (Fig. 6D), indicating that a rescue phenotype can be achieved by the both ways and both miRNAs can be potentially used in clinical treatment.

Discussion

MDR and disease relapse is a challenging clinical problem in the treatment of leukaemia. Although standard chemotherapy results in a higher CR rate, for example 56–74% CR rate in acute myeloid leukaemia (AML) patients, a number of patients still experience

Fig. 6 Effect of increased levels of miR-27a and miR-331–5p on the sensitivity of K562/DOX cells to DOX. Drug resistance of K562/DOX1 cells transfected with either miR-27 or/and miR-331–5p, as well as the resistance of scrambled oligonucleotides (NC)-transfected K562/DOX1 cells, were tested by applying increasing concentrations of the tested drugs and measuring the surviving cells with the MTT cell proliferation assay at 48 hrs after treatment. (**A**) Elevated levels of miR-27a or miR-331–5p in the mimic-transfected K562/DOX1 cells sensitized these cells to DOX. (**B**) The bar chart shows the IC50 value decreased with the two miRNAs transfected into K562/DOX1 cells. $^*P < 0.05$, compared with that of NC. (**C**) The transfection of K562/DOX1 cells with siRNAs against the MDR1 mRNA resulted in a strong decrease in the levels of the endogenous protein P-gp. Error bars represent standard deviation. (**D**) A rescue phenotype can be achieved by either overexpressed miRNAs or down-regulated MDR1. $P < 0.01$, compared with that of K562/WT cells.

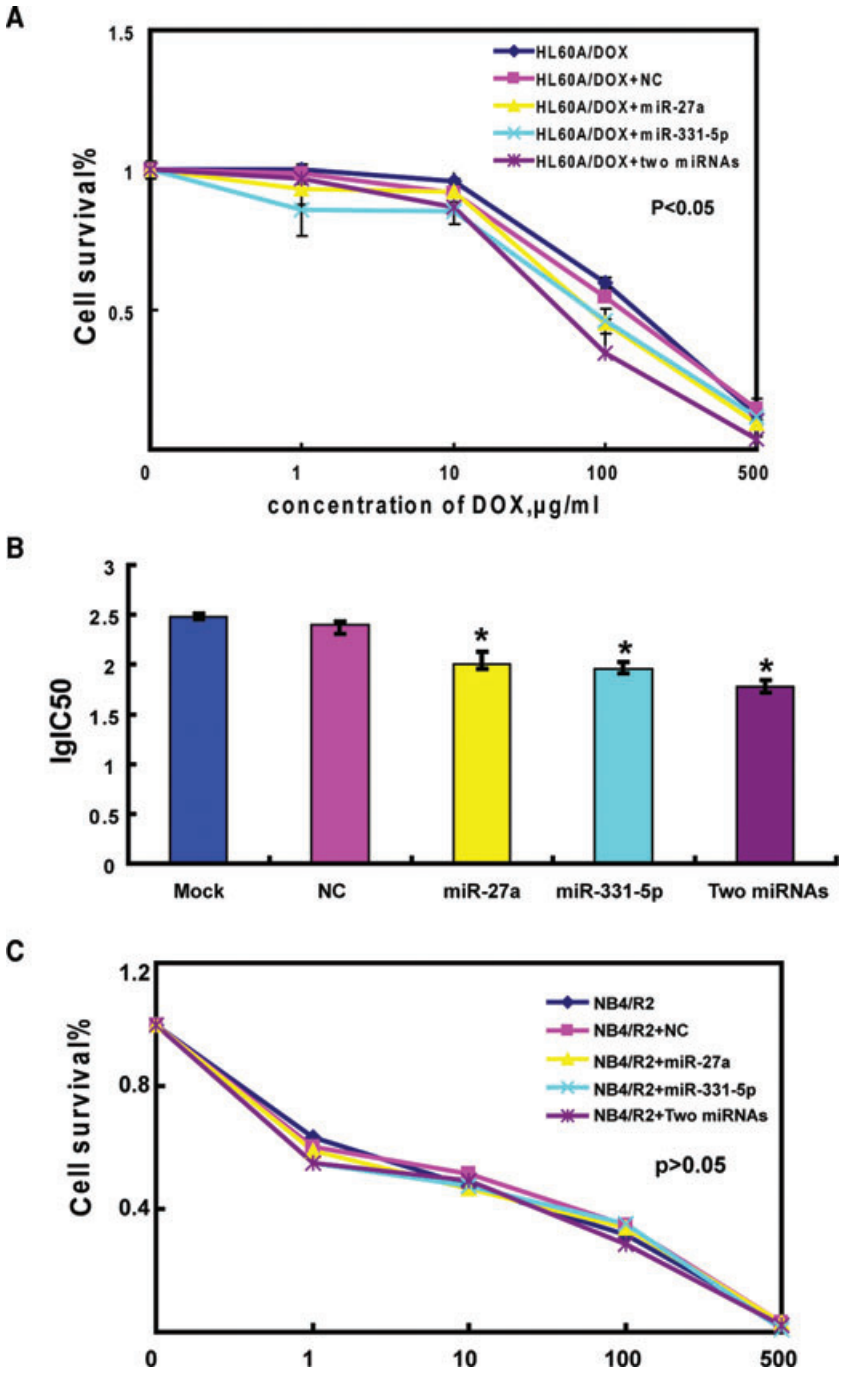


Fig. 7 Effect of miR-27a and miR-331–5p on the sensitivity of HL60/DOX and NB4/R2 cells. (**A**) Drug resistance of HL60/DOX cells transfected with either miR-27a or/and miR-331–5p, as well as the resistance of scrambled oligonucleotides (NC)-transfected HL60/DOX cells, were tested by MTT assay at 48 hrs after treatment. (**B**) IC50 value decreased with the two miRNAs transfected into HL60/DOX cells. Error bars represent standard deviation. $^*P < 0.05$, compared with that of NC. (**C**) Drug resistance of NB4/R2 cells transfected with either miR-27a or/and miR-331–5p, as well as the resistance of scrambled oligonucleotides (NC)-transfected NB4/R2 cells, were tested by MTT assay at 48 hrs after treatment. $P > 0.05$, compared with that of NC.

relapse. Relapsed disease is frequently refractory to chemotherapy and exhibits MDR [26]. Several mechanisms may account for this phenomenon, including failure of cells to undergo apoptosis in response to chemotherapy or failure of the drug to reach and/or affect its intracellular target. Many investigations have focused on the relationship between P-gp expression and tumour progression. It was reported that expression level of P-gp increased after treatment at the time of relapse [27–30]. Despite the well-established role of P-gp in leukaemia progression and drug resistance to chemotherapeutic drugs, the molecular mechanism for

regulation of P-gp is still not fully understood. Recently, increasing studies have indicated that aberrant miRNA expression is involved in the response of tumour cells to chemotherapeutic agents and miRNAs have been implicated in the anticancer drug-resistance phenotype [31–33]. In this study, we documented the miRNA profile in drug-resistant leukaemia cells to identify and characterize a mechanistic connection between miRNA dysregulation with the established multidrug-resistant phenotype in leukaemia. Importantly, we elucidated that two miRNAs, miR-27a and miR-331–5p, were involved in the resistance to the chemotherapeutic drug DOX in the leukaemia cell line K562. Their down-regulated expressions were also found in clinical relapse samples. The study provides new evidence about the roles of miRNAs in the MDR development of tumour cells and suggests the potential relationship between miRNAs and leukaemia relapse. Further study on the inverse correlation of P-gp expression and the both miRNAs with large numbers of patients would be interesting and necessary.

MiR-27a has been shown to be involved in the tumorigenesis of several cancers. Mertens-Talcott reported that miR-27a exhibits oncogenic activity through regulation of ZBTB10, which, in turn, results in overexpression of Sp proteins and Sp-dependent genes that are important for cell survival and angiogenesis in breast cancer cells [34]. An anticancer agent, Methyl 2-cyano-3,11-dioxo-18b-olean-1,12-dien-30-oate, was found to suppress tumour activity due to repression of oncogenic miR-27a [35]. Up-regulation of miR-27a also resulted in overexpression of P-gp in human ovarian cancer and cervical carcinoma multidrug-resistant cell lines [16]. However, in these two cancer cell lines, miR-27a did not directly bind to the 3′-UTR of the *MDR1* gene and did not inhibit P-gp protein expression, which is in contrast with our results. Similar results were observed for miR-451, which was involved in activating the expression of P-gp in ovarian cancer and cervical carcinoma but inhibited protein expression in breast cancer [16]. We propose that these miRNAs might regulate drug-resistance factors differently depending on the particular malignancy.

Only very few studies have addressed the role of miR-331–5p in carcinogenesis [2, 36]. There have been no reports about the role of miR-331–5p in drug resistance, especially in leukaemia. In this study, we determined that miR-331–5p was involved in DOX resistance as a drug-resistance factor. To our knowledge, this is the first report associating miR-331–5p with anticancer drug resistance. At the time of the preparation of this manuscript, a study reported that miR-138 might mediate drug resistance, at least in part, through regulation of MDR1 in the vincristine-induced, multidrug-resistant leukaemia cell line HL60/VCR [37]. However, we did not find down-regulation of miR-138 using miRNA microarray. This may be due to different cell lines and different drugs used, further supporting our hypothesis that miRNAs play different roles in particular malignancies.

Notably, we also found that miR-331–5p and miR-27a were relevant to the treatment outcome *in vivo* and that they might be involved in the relapse of lymphocytic leukaemia and myeloid leukaemia. This finding suggests that correction of the altered expression of miRNA may be used in therapeutic strategies aiming to overcome cancer cell resistance. Our previous study showed that an ‘miRNA cascade’ was associated with central nervous system relapse in acute lymphocytic leukaemia (ALL) [38]. Gallardo *et al.* showed that miR-34a could be a prognostic marker of relapse in surgically resected non-small-cell lung cancer [39]. Patients with low miR-135a expression had a higher probability of relapse and a shorter disease-free survival in classic Hodgkin lymphoma [40]. Down-regulation of miR-221 was associated with clinico-pathological parameters, including the Gleason score and clinical recurrence during follow-up in aggressive prostate cancer [41]. However, the key determinants of the role of miRNA in MDR and cancer relapse remain largely unknown. Our results demonstrate that miR-331–5p and miR-27a might be involved in the drug resistance, as well as the disease relapse, caused by MDR. However, it must be pointed out that single miRNA, that is miR-331–5p or miR-27a, as a clinical therapeutic target might not be sufficient to inhibit MDR expression in drug-resistant cells. Combination of two or more miRNAs might be more efficient for cancer therapy.

In conclusion, this study showed that the miRNA expression profile was associated with DOX resistance in K562 human leukaemia cells. In addition, we showed that the expression of miR-331–5p and miR-27a was inversely correlated with MDR1 expression. Transfection of exogenous miR-27a or miR-331–5p, or a combination of these two miRNAs, down-regulated *MDR1* and increased sensitivity of the K562-resistant cancer cells to DOX. Furthermore, we found that the expression level of miR-27a or miR-331–5p was lower in patients with relapsed lymphocytic leukaemia and myeloid leukaemia compared to untreated patients, indicating that these two miRNAs might be related to clinical relapse. These results may be useful for further studies of MDR prediction in patients and might provide a strong rationale for the development of miRNA-based therapeutic strategies aiming to overcome drug-resistant leukaemia.

Acknowledgements

We thank the following investigators and hospitals that provided samples for the analysis: Dr. Hai-Xia Guo at the Second Affiliated Hospital of Sun Yat-sen University, Li-Bing Huang at the First Affiliated Hospital of Sun Yat-sen University and Li-Ming Tu at Guangdong General Hospital. This work was supported by the National Natural Science Foundation of China (No. 30672254 and 30872784 to Y.Q.C., No. 30772498 to P.Z.), National High-Tech Program (863, No. 2008AA02Z106 to Y.Q.C.) and National Science and Technology Department and Guangdong Province (2011CB811301 to L.H.Q. and 2009ZX09103-641 to Y.Q.C.).

Conflict of interest

The authors declare no competing financial interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Characteristic of patients

Table S2 Primer sequence for vector construction and miRNA qRT-PCR detection

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References

1. **Kaspers GJ, Veerman AJ, Pieters R, *et al.*** *In vitro* cellular drug resistance and prognosis in newly diagnosed childhood acute lymphoblastic leukemia. *Blood*. 1997; 90: 2723–9.
2. **Gaur A, Jewell DA, Liang Y, *et al.*** Characterization of microRNA expression levels and their biological correlates in human cancer cell lines. *Cancer Res*. 2007; 67: 2456–68.
3. **Glasspool RM, Teodoridis JM, Brown R.** Epigenetics as a mechanism driving polygenic clinical drug resistance. *Br J Cancer*. 2006; 94: 1087–92.
4. **Iwasa Y, Nowak MA, Michor F.** Evolution of resistance during clonal expansion. *Genetics*. 2006; 172: 2557–66.
5. **Roberti A, La Sala D, Cinti C.** Multiple genetic and epigenetic interacting mechanisms contribute to clonally selection of drug-resistant tumours: current views and new therapeutic prospective. *J Cell Physiol*. 2006; 207: 571–81.
6. **Goasguen JE, Dossot JM, Fardel O, *et al.*** Expression of the multidrug resistance-associated P-glycoprotein (P-170) in 59 cases of de novo acute lymphoblastic leukemia: prognostic implications. *Blood*. 1993; 81: 2394–8.
7. **Ivy SP, Olshefski RS, Taylor BJ, *et al.*** Correlation of P-glycoprotein expression and function in childhood acute leukemia: a children's cancer group study. *Blood*. 1996; 88: 309–18.
8. **Leonard GD, Fojo T, Bates SE.** The role of ABC transporters in clinical practice. *Oncologist*. 2003; 8: 411–24.
9. **Bartel DP.** MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004; 116: 281–97.
10. **Fojo T.** Multiple paths to a drug resistance phenotype: mutations, translocations, deletions and amplification of coding genes or promoter regions, epigenetic changes and microRNAs. *Drug Resist Updat*. 2007; 10: 59–67.
11. **Sevignani C, Calin GA, Siracusa LD, *et al.*** Mammalian microRNAs: a small world for fine-tuning gene expression. *Mamm Genome*. 2006; 17: 189–202.
12. **Yang H, Kong W, He L, *et al.*** MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN. *Cancer Res*. 2008; 68: 425–33.
13. **Xia L, Zhang D, Du R, *et al.*** miR-15b and miR-16 modulate multidrug resistance by targeting BCL2 in human gastric cancer cells. *Int J Cancer*. 2008; 123: 372–9.
14. **Kovalchuk O, Filkowski J, Meservy J, *et al.*** Involvement of microRNA-451 in resistance of the MCF-7 breast cancer cells to chemotherapeutic drug doxorubicin. *Mol Cancer Ther*. 2008; 7: 2152–9.
15. **Liang Z, Wu H, Xia J, *et al.*** Involvement of miR-326 in chemotherapy resistance of breast cancer through modulating expression of multidrug resistance-associated protein 1. *Biochem Pharmacol*. 2010; 79: 817–24.
16. **Zhu H, Wu H, Liu X, *et al.*** Role of MicroRNA miR-27a and miR-451 in the regulation of MDR1/P-glycoprotein expression in human cancer cells. *Biochem Pharmacol*. 2008; 76: 582–8.
17. **Davies GF, Juurlink BH, Harkness TA.** Troglitazone reverses the multiple drug resistance phenotype in cancer cells. *Drug Des Devel Ther*. 2009; 3: 79–88.
18. **Silva J, Barrandon O, Nichols J, *et al.*** Promotion of reprogramming to ground state pluripotency by signal inhibition. *PLoS Biol*. 2008; 6: e253.
19. **Xie X, Lu J, Kulbokas EJ, *et al.*** Systematic discovery of regulatory motifs in human promoters and 3' UTRs by comparison of several mammals. *Nature*. 2005; 434: 338–45.
20. **Eisen MB, Spellman PT, Brown PO, *et al.*** Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA*. 1998; 95: 14863–8.
21. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods*. 2001; 25: 402–8.
22. **Grandjean F, Bremaud L, Verdier M, *et al.*** Sequential gene expression of P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP) and lung resistance protein: functional activity of P-gp and MRP present in the doxorubicin-resistant human K562 cell lines. *Anticancer Drugs*. 2001; 12: 247–58.
23. **Baran Y, Gur B, Kaya P, *et al.*** Upregulation of multi drug resistance genes in doxorubicin resistant human acute myelogeneous leukemia cells and reversal of the resistance. *Hematology*. 2007; 12: 511–7.
24. **Lewis BP, Burge CB, Bartel DP.** Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*. 2005; 120: 15–20.
25. **Yoon S, De Micheli G.** Computational identification of microRNAs and their targets. *Birth Defects Res C Embryo Today*. 2006; 78: 118–28.
26. **List AF.** Role of multidrug resistance and its pharmacological modulation in acute myeloid leukemia. *Leukemia*. 1996; 10: 937–42.
27. **Fisher GA, Sikic BI.** Clinical studies with modulators of multidrug resistance. *Hematol Oncol Clin North Am*. 1995; 9: 363–82.
28. **Leith CP, Kopecky KJ, Godwin J, *et al.*** Acute myeloid leukemia in the elderly: assessment of multidrug resistance (MDR1) and cytogenetics distinguishes biologic subgroups with remarkably distinct responses to standard chemotherapy. A Southwest Oncology Group study. *Blood*. 1997; 89: 3323–9.
29. **McGrogan BT, Gilmartin B, Carney DN, *et al.*** Taxanes, microtubules and chemoresistant breast cancer. *Biochim Biophys Acta*. 2008; 1785: 96–132.
30. **Stewart J, Gorman NT.** Multi-drug resistance genes in the management of neoplastic disease. *J Vet Intern Med*. 1991; 5: 239–47.

31. **Cho WC, Chow AS, Au JS.** Restoration of tumour suppressor hsa-miR-145 inhibits cancer cell growth in lung adenocarcinoma patients with epidermal growth factor receptor mutation. *Eur J Cancer*. 2009; 45: 2197–206.
32. **Koschny R, Walczak H, Ganten TM.** The promise of TRAIL–potential and risks of a novel anticancer therapy. *J Mol Med*. 2007; 85: 923–35.
33. **Leith CP, Kopecky KJ, Chen IM, *et al.*** Frequency and clinical significance of the expression of the multidrug resistance proteins MDR1/P-glycoprotein, MRP1, and LRP in acute myeloid leukemia: a Southwest Oncology Group Study. *Blood*. 1999; 94: 1086–99.
34. **Mertens-Talcott SU, Chintharlapalli S, Li X, *et al.*** The oncogenic microRNA-27a targets genes that regulate specificity protein transcription factors and the G2-M checkpoint in MDA-MB-231 breast cancer cells. *Cancer Res*. 2007; 67: 11001–11.
35. **Chintharlapalli S, Papineni S, Abdelrahim M, *et al.*** Oncogenic microRNA-27a is a target for anticancer agent methyl 2-cyano-3,11-dioxo-18beta-olean-1, 12-dien-30-oate in colon cancer cells. *Int J Cancer*. 2009; 125: 1965–74.
36. **Zanette DL, Rivadavia F, Molfetta GA, *et al.*** miRNA expression profiles in chronic lymphocytic and acute lymphocytic leukemia. *Braz J Med Biol Res*. 2007; 40: 1435–40.
37. **Zhao X, Yang L, Hu J, *et al.*** miR-138 might reverse multidrug resistance of leukemia cells. *Leuk Res*. 2010; 34: 1078–82.
38. **Zhang H, Luo XQ, Zhang P, *et al.*** MicroRNA patterns associated with clinical prognostic parameters and CNS relapse prediction in pediatric acute leukemia. *PLoS One*. 2009; 4: e7826.
39. **Gallardo E, Navarro A, Vinolas N, *et al.*** miR-34a as a prognostic marker of relapse in surgically resected non-small-cell lung cancer. *Carcinogenesis*. 2009; 30: 1903–9.
40. **Navarro A, Diaz T, Martinez A, *et al.*** Regulation of JAK2 by miR-135a: prognostic impact in classic Hodgkin lymphoma. *Blood*. 2009; 114: 2945–51.
41. **Spahn M, Kneitz S, Scholz CJ, *et al.*** Expression of microRNA-221 is progressively reduced in aggressive prostate cancer and metastasis and predicts clinical recurrence. *Int J Cancer*. 2010; 127: 394–403.