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ORIGINAL ARTICLE

miR-181a sensitizes resistant leukaemia HL-60/Ara-C cells to Ara-C by inducing apoptosis

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Abstract
Background Ara-C is one of the most commonly used drugs in the treatment of AML. However, the development of drug resistance always prevented its further use. It has been shown that miR-181a is associated with the clinical outcome of AML patients. Here, we investigated the possible role of miR-181a in AML Ara-C resistance.
Methods miR-181a expression was measured by real-time PCR. Cell viability was detected by MTT assay. Protein expressions were measured by western blotting. Caspase activity was examined by florescence assay.
Results We found that miR-181a expression was down-regulated in the Ara-C-resistant cell line HL-60/Ara-C compared with its parental cell line HL-60. Overexpression of miR-181a in HL-60/Ara-C cells sensitized the cells to Ara-C treatment. Furthermore, Bcl-2 was confirmed as a direct miR-181a target by immunoblot analysis and reporter gene assays. Knockdown of Bcl-2 mimicked the effect of enforced miR-181a expression by reducing cell viability. In addition, the apoptosis pathway was activated by cytochrome C release and caspase 9/caspase 3 activation after miR-181a overexpression.
Conclusions This study for the first time demonstrated that downregulation of miR-181a and upregulation of Bcl-2 in leukaemia cells confer resistance to Ara-C-based therapy. These results suggest that restoration of miR-181a expression might provide a promising therapeutic in drug resistance of leukaemia.

Keywords Chemoresistance · miR-181a · Ara-C · Acute myeloid leukaemia · Bcl-2

Introduction

Ara-C is an effective agent in the treatment of leukaemia, particularly in acute myeloid leukaemia (Tallman et al. 2005). It is a nucleoside analogue antimetabolite, which exerts its function through its derivative Ara-CTP. Ara-CTP incorporates into DNA, which induces DNA double-strand breaks and subsequent cell death (Fernandez-Calotti et al. 2005). Although Ara-C-based chemotherapeutic treatments achieve dramatic remissions, the majority of patients relapse with drug-resistant disease. The reasons underlying the drug resistance to Ara-C attract extensive laboratory investigations. The deoxycytidine kinase (dCK) deficiency has been believed to be one of the mechanisms of Ara-C resistance (Bhalla et al. 1984). Other mechanisms include the loss of function of hENT1/SLC29A1 (a nucleoside transporter used by Ara-C) (Cai et al. 2008), activation of NF-κB, telomerase activity and Fas expression (Kanno et al. 2007) and enhanced degradation of Ara-C (Momparler and Laliberte 1990). However, the detailed molecular mechanisms that contribute to Ara-C resistance are still not fully understood.

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MicroRNAs (miRNA) are a class of noncoding RNAs that regulate target genes at a post-transcriptional level. Although only about 750 microRNAs were cloned, they play a crucial role in many biological processes including development, cancerogenesis, cell survival and apoptosis (He and Hannon 2004). Increasing findings have shown that microRNAs are involved in tumour cells' resistance to chemotherapeutic agents (Blower et al. 2008). But the role of microRNAs in drug resistance in AML is largely unknown.

Recent study indicates that miR-181a expression could predict the clinical outcome of AML patients (Schwind et al. 2010; Marcucci et al. 2011). The patients with higher miR-181a expression had a higher complete remission (CR) rate, longer overall survival and a trend for longer disease-free survival. Based on the therapeutic role of Ara-C in AML and the prognostic significance of miR-181a in AML, we hypothesized that dysregulation of miR-181a may be involved in Ara-C resistance in AML. Resistant cell line is a useful tool to study the genotype changes in vitro. In this study, we take advantage of HL-60/Ara-C cell line to study the Ara-C resistance in AML. We show that miR-181a is lowly expressed in Ara-C-resistant leukaemia HL-60 cell line. Furthermore, miR-181a negatively regulates Bcl-2 expression and sensitizes Ara-C resistance cells to apoptosis by activating caspase cascade. These results provide us important information on the molecular mechanisms of Ara-C resistant in AML.

Materials and methods

Cell culture

The HL-60 cell line and the HL-60/Ara-C-resistant cell line were a kind gift from Dr. Su Liping (Shanghai Ruijin Hospital). The cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% foetal bovine serum, 100 g/ml L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. The HEK293T cells were grown in DMEM that contained 10% foetal bovine serum, 100 g/ml L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin.

Cell viability assay

Cells were seeded into 96-well plates in RPMI-1640 medium containing 10% FBS. After 24 h, the cells were treated with serial dilutions of Ara-C. Approximately 72 h after Ara-C treatment, MTT was added to a final concentration of 0.5 mg/ml, and the cells were incubated for 4 h at 37°C. The optical density was read at 570 nm using a microplate spectrophotometer. Each experiment was carried out in triplicate and repeated three times.

Real-time PCR analysis

The total cellular RNA was isolated using a RecoverAll kit (Ambion Inc., Austin, Tx, USA) for both the miR-181a and Bcl-2 mRNA analyses according to the manufacturer's instructions. To detect miR-181a expression, a TaqMan microRNA Assay (Applied Biosystems, Foster City, CA, USA) was performed as described (Bai et al. 2011) on an Applied Biosystems ABI 7500 Real Time PCR system. U6 was used as a control. To detect Bcl-2 mRNA expression, real-time PCR was performed using a Quantitect SYBR Green PCR Kit (Qiagen). β-Actin was used to normalize the Bcl-2 mRNA expression level. The forward and reverse primers sequences used for Bcl-2 mRNA amplification were 5′-ATGGCGCACGCTGGGAGAACAGGGT-3′ and 5′-ACAGCCAGGAGAAATCAAACAGAGGC-3′, respectively. The β-actin forward and reverse primers were 5′-CAGAGCCTCGCCTTTGCC-3′ and 5′-GTCGCCCACATAGGAATC-3′, respectively. All of the real-time PCR assays were performed in triplicate and repeated three times.

Plasmid constructs and retroviral transduction

A 450-bp DNA fragment containing the miR-181a precursor was PCR-amplified from human genomic DNA (Roche Applied science) with the following primers: forward: 5′-AGATCTAGCCCAATATCGGCCATGTT-3′ and reverse: 5′-CTCGAGAGAAAGTCCTGGTGTGTCCA-3′; the XhoI and BglII recognition sites are underlined. The amplicon was cloned into a modified MSCV-PGK-GFP vector (Clotech). To generate cell lines that stably expressed miR-181a in either HL-60 or HL-60/Ara-C, 10 μg expression vector or empty vector with 10 μg packing plasmid (gag/pol and VSVG) was incubated with Fugene6 (Roche) for 15 min at room temperature and subsequently added to the HEK293T cells. The viral supernatant was harvested after 48 h, and the cells were treated by a spin infection with retroviral supernatant (1 ml supernatant per 1×10^6 cells plus polybrene) in 6-well plates that were pre-coated with retronectine (Takara); after 24 h, the cells were centrifuged at $1{,}500 \times g$ for 30 min. The GFP-positive cells were approximately 60–75% and sorted by flow cytometry.

Transfection and luciferase activity assay

A Bcl-2 3′UTR luciferase reporter was created by inserting full-length human Bcl-2 3′UTR into the XhoI and NotI sites in the psi-CHECK2 vector (Promega) downstream from the renilla luciferase coding sequence. The Bcl-2 3′UTR was PCR-amplified from human genomic DNA with the following primers: forward 5′-TCGCTCGAGAGTC AACATGCCTG CCCCAAAC-3′ and reverse 5′-TCGGCGGCCGCCAGTATCTCACACTGTACTTTAT-3′.

Mutations within the putative miR-181a binding sites were created with a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The mutagenic primers were forward 5′-ATGTTAGAAGCAATGACCCTATAT AAAAGCCT-3′ and reverse 5′-AGGCTTTTATATA GGGTCATTGCTTCTAACAT-3′. The HL-60 cells were plated in 96-well plates at 5,000 cells per well the day before transfection. Transfection was performed in triplicate with Fugene6 (Roche) and 100 ng of the plasmid mixture (90 ng of the miR-181a expression vector and 10 ng of the reporter vector). Luciferase assays for both firefly and renilla luciferase were performed 48 h after transfection with a Dual-Glo Luciferase assay kit (Promega). Luminescence was quantified using a Tecan Spectrafluor Plus machine. The renilla luciferase readings were normalized to the firefly luciferase activity in the corresponding well.

Western blot analysis

Expression levels of Bcl-2, caspase 3, caspase 9 and cytochrome C were determined by western blot. Briefly, approximately 30 μg of protein extracts was loaded on an 8% SDS-PAGE gel and subsequently transferred to a polyvinylidene difluoride membrane (PVDF; Biorad). The membranes were blocked with 5% milk and incubated with primary antibody overnight at 4°C, followed by incubation with secondary antibody. The primary antibodies for Bcl-2, caspase 3, caspase 9 and cytochrome C were purchased from Cell Signaling Technology; the β-actin antibody was purchased from Sigma. Band detection via enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL; Pierce biotechnology Inc., Rockford, IL, USA). The protein bands were quantified using ImageJ 1.33 software (NIH), and the data were normalized to β-actin.

Cytosolic cytochrome C fractions were obtained as described before (Ma et al. 2009). Briefly, cells were washed with PBS and suspended in a buffer containing (in mM) 250 sucrose, 20 HEPES, pH 7.5, 10 KCl, 1.5 $MgCl_2$, 1 EGTA, 1 EDTA, 1 DTT and protease inhibitors. The supernatant was incubated on ice for 30 min and homogenized by passing through 25-gauge needle. Homogenates were centrifuged at 800xg for 10 min, and the suspension was centrifuged at 20,000$\times g$ for 20 min at 4°C. The supernatant contained cytosolic fractions.

siRNA transfection

A SignalSilence Bcl-2 siRNA kit was purchased from Cell Signaling Technology (CST), and the transfection was performed according to the manufacturer's protocol. The cells were prepared for further analysis 48 h after transfection. The transfection efficiency was evaluated by fluorescence microscopy by calculating the percentage of fluorescein/labelled cells. The transfection efficiency was approximately 80%.

Caspase activity assay

Caspase activity was assayed using Caspase Colorimetric Assay Kit (KeyGEN, Nan Jing, China). Briefly, cells were lysed in lysis buffer on ice for 20 min. After centrifugation, the supernatants were incubated with the caspase substrate at 37°C for 4 h in a reaction buffer according to the manufacturer's instructions and then read on a 96-well plate reader at 405 nm. The percentage of A405 values for the samples versus those for control samples indicated the percentage of caspase activity.

Statistical analysis

The data are presented as the mean ± S.E. Statistical analysis was performed with SPSS 13.0 software. One-way ANOVA with post hoc Tukey's test was performed for experiments that involved more than two groups, while Student's *t* test was performed for comparisons between the two groups. A $P < 0.05$ was considered to be significant.

Results

HL-60/Ara-C cells are resistant to Ara-C

The HL-60 cell line is a commonly used cell model for the study of AML. We take advantage of a resistant cell line HL-60/Ara-C to study the Ara-C resistance in AML. The cytotoxicity of Ara-C was determined using the 3-(4,5 dimethylthiazol-2-yl-)5 diphenyltetrazolium bromide (MTT) assay. HL-60/Ara-C cells showed a strong resistance to Ara-C at the concentration of 1 and 10 μM compared with the parental HL-60 cells (Fig. 1a, $P < 0.001$).

miR-181a is downregulated in HL-60/Ara-C cells

To evaluate the role of miR-181a in the Ara-C resistance, we first tested the miR-181a expression in HL-60 cells as well as in HL-60/Ara-C cells. The results obtained by real-time PCR confirmed that the expression of miR-181a in HL-60/Ara-C was almost 20% of that in HL-60 (Fig. 1b, lane 2, $P < 0.001$), suggesting that miR-181a may play a role in the Ara-C resistance in HL-60 cells.

Overexpression of miR-181a in drug resistance cells sensitized the cells to Ara-C

To further validate the effect of miR-181a on drug resistance, cells were overexpressed with miR-181a and treated

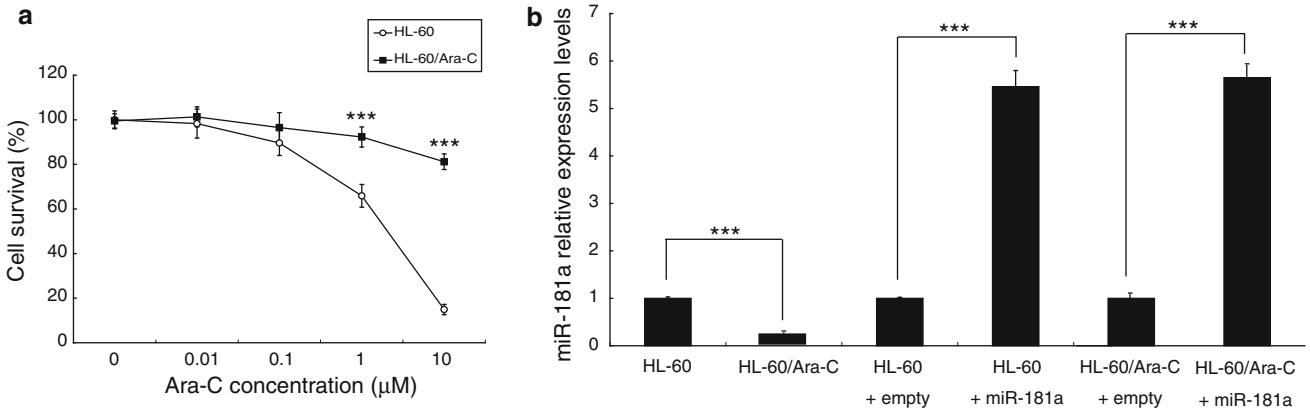

Fig. 1 miR-181a expression was decreased in Ara-C resistance cells compared with its parental HL-60 cells. **a** A survival curve of HL-60 and HL-60/Ara-C cells. Cell viability was evaluated by MTT assay. **b** miR-181a expression was examined by real-time PCR. HL-60 and HL-60/Ara-C cells were transduced with MSCV-miR-181a vector or an empty vector. The results are expressed as the mean ± SEM from three independent experiments. ***$P < 0.001$

with various doses of Ara-C. Real-time PCR revealed that miR-181a was highly expressed in both HL-60 (Fig. 1b, lane 4, $P < 0.001$) and HL-60/Ara-C (Fig 1b, lane 6, $P < 0.001$) cells after stably transduced with an MSCV-miR-181a vector, while no change was observed in both cells after stably transduced with empty vector [Fig. 1b, lane 3 (HL-60), lane 5 (HL-60/Ara-C), $P > 0.05$]. Overexpression of miR-181a was associated with significantly decreased survival of the HL-60/Ara-C cells at Ara-C concentrations higher than 1 μM (Fig. 2b, $P < 0.05$). However, overexpression of miR-181a in HL-60 cells did not affect the response to Ara-C treatment (Fig. 2a, $P > 0.05$).

Bcl-2 is a direct target of miR-181a

Bcl-2 3′UTR contains a highly conserved 8 mer site complementary to the seed region of the miR-181a (TargetScan Release 5.2). We thus hypothesized that Bcl-2 is a direct miR-181a target. The HL-60 cells were cotransfected with Bcl-2-3′UTR luciferase reporter and miR-181a. Transfections with control vector were performed in parallel. As shown in Fig 3b, miR-181a significantly reduced luciferase activity of the Bcl-2-3′UTR reporter ($P < 0.001$). However, transfection of the Bcl-2 3′UTR mutant did not affect reporter activity ($P > 0.05$). We also assessed whether miR-181a regulated Bcl-2 expression in HL-60/Ara-C cells. As shown in Fig. 3c, d and e, both Bcl-2 mRNA and protein levels were dramatic decreased after the overexpression of miR-181a ($P < 0.001$). These results show that Bcl-2 is a target of miR-181a.

miR-181a regulates caspase-dependent cell death through Bcl-2

The regulation of apoptosis by the Bcl-2 protein is highly complex. We hypothesized that overexpression of miR-181a may activate caspase cascade by reducing the Bcl-2 protein level, resulting in HL-60/Ara-C cells sensitive to Ara-C. To test the downstream factors of Bcl-2, we examined the activation of caspase cascade and release of

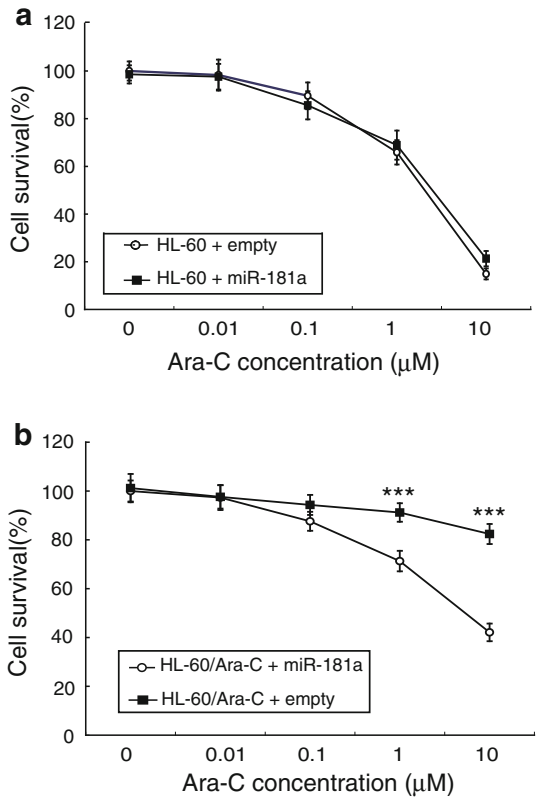

Fig. 2 Overexpression of miR-181a in drug resistance cell sensitizes the cells to Ara-C. **a** HL-60 cells were transduced with MSCV-miR-181a or an empty vector and were treated with various doses of Ara-C. **b** The HL60/Ara-C cells were transduced with an MSCV-miR-181a vector or an empty vector and were treated with various doses of Ara-C. The cell viability was detected by MTT assay. The results are expressed as the mean ± SEM from three independent experiments. ***$P < 0.001$

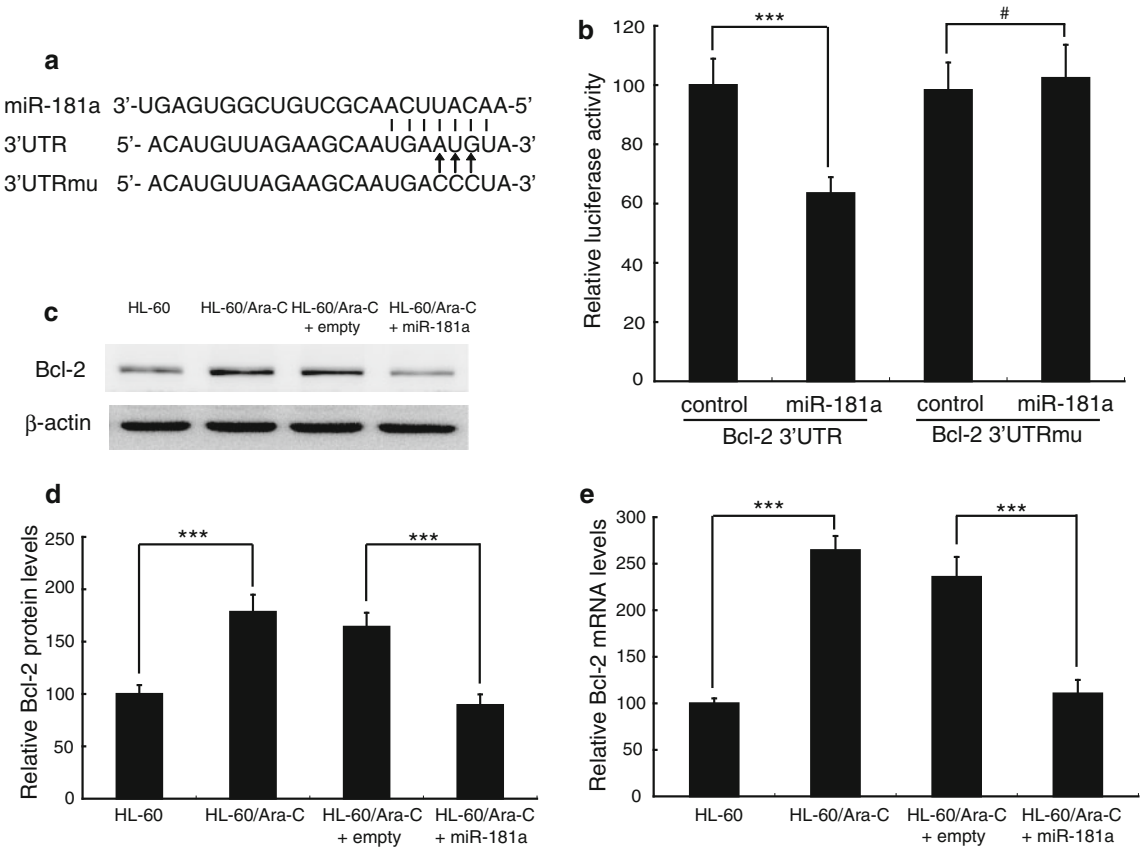

Fig. 3 Bcl-2 is a direct target of miR-181a. **a** A nucleotide comparison between the miR-181a seed sequence and the Bcl-2 3′UTR sequence. Three nucleotides in the miR-181a seed region were mutated with PCR site-directed mutagenesis. The *arrows* show the mutated nucleotides. **b** The effect of miR-181a on Bcl-2 was assessed with the luciferase reporter system. The miR-181a expression vector, together with the luciferase reporter vector or control vector, was cotransfected into the HL-60 cells. The data showed that miR-181a binds to the Bcl-2 3′UTR, resulting in reduced luciferase expression, while it does not repress luciferase activity when cotransfected with Bcl-2 3′UTR mutant construct. Data are mean ± SEM from three independent experiments. $^{***}P < 0.001$, $^{\#}P > 0.05$. **c**, **d** Cell lysates were immunoblotted with anti-Bcl-2 antibody. *β*-Actin was used as loading control. Blots were representative of four independent experiments. **e** Bcl-2 mRNA was determined by real-time PCR. Relative expression of Bcl-2 mRNA was calculated by ΔΔCT methods. The results are expressed as mean ± SEM from three independent experiments. $^{***}P < 0.001$

cytochrome C. As shown in Fig. 4, the obvious decrease in cytochrome C in the cytosolic fractions (Fig. 4a) and inactivation of caspase-3 and caspase-9 activity (Fig. 4a, b) were observed in HL-60/Ara-C cells, corresponding to the increased expression of Bcl-2. In contrast, the significant increase of cytochrome C expression in the cytosolic fractions (Fig. 4a) and activation of caspase-3 and caspase-9 activity (Fig. 4a, b) were observed in HL-60/Ara-C cells after transduced with miR-181a.

Bcl-2 plays a key role in HL-60/Ara-C resistance

To definitively confirm the role of Bcl-2 in the Ara-C resistance of leukaemia, we used Bcl-2 siRNA to knockdown the expression of Bcl-2 in HL-60/Ara-C cells. As shown in Fig. 5a, the expression of Bcl-2 was significantly decreased after transfection of Bcl-2 siRNA. Furthermore, we measured the changes in cytochrome C, caspase 3 and caspase 9 to determine the role of miR-181a in the apoptosis pathway through Bcl-2. We found that these proteins were activated after Bcl-2 siRNA treatment (Fig. 5a, b). More interestingly, the HL-60/Ara-C cells that were pre-treated with Bcl-2 siRNA had similar survival pattern as miR-181a overexpression (Fig 5c). These results implicate that Bcl-2 plays an important role in Ara-C resistance in AML.

Discussion

In this study, we report here for the first time that Ara-C-induced drug resistance is associated with downregulation of miR-181a in the leukaemia cell line HL-60. miR-181a may regulate the death of Ara-C-resistant leukaemia cells by targeting Bcl-2 expression and causing subsequent changes in the apoptosis pathway.

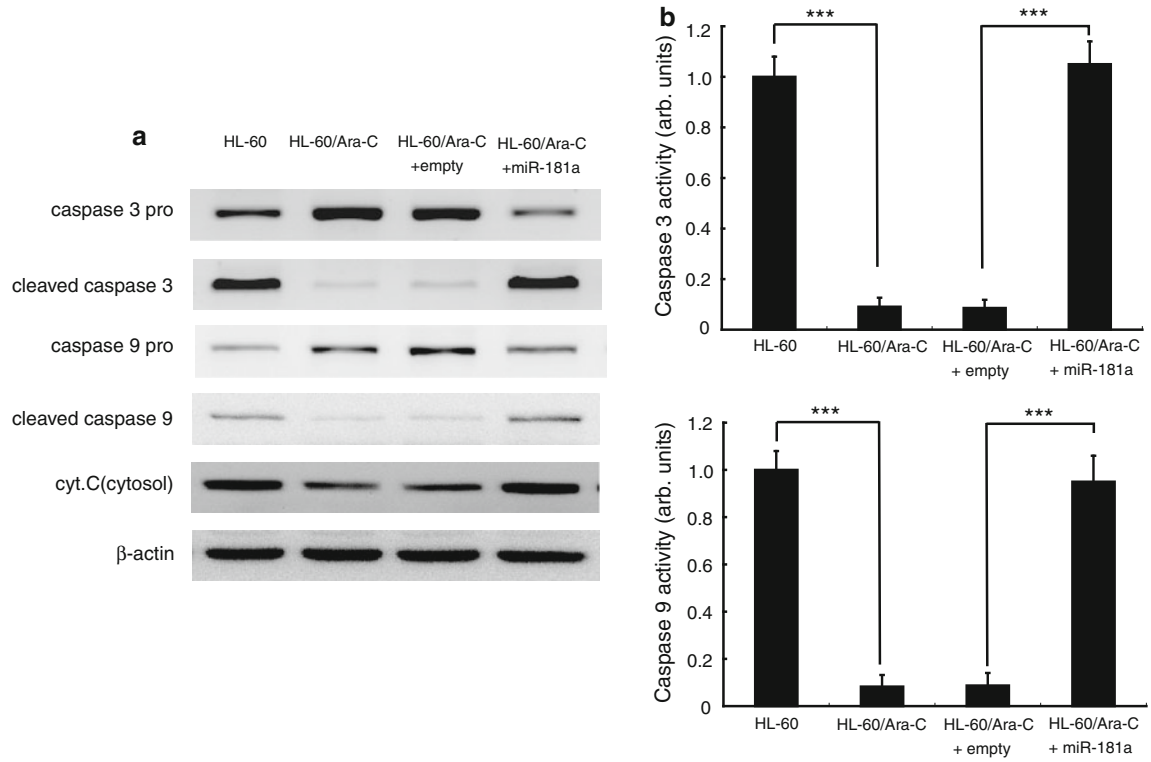

Fig. 4 Overexpression of miR-181a regulates cytochrome C release and caspase pathway by Bcl-2. **a** Representative western blots showing caspase 3, caspase 9 and cytosolic cytochrome C protein levels in HL-60 and HL-60/Ara-C cells as well as in HL-60/Ara-C cells that transduced with an empty vector or the MSCV-miR-181a vector when cells were exposed to 1 μM Ara-C. Total cell lysates or cytosolic fractions were prepared and the caspase pathway proteins were detected. **b** Activity of caspase 3 and caspase 9 was decreased in HL-60/Ara-C cells compared with HL-60 cells; however, activity of caspase 3 and caspase 9 was increased in HL-60/Ara-C cells after overexpression of miR-181a

Recently, there is increasing interest in understanding the role of miR-181 in cancers. It has been shown that miR-181a may function as oncogene or tumour suppressor. miR-181a expression is elevated in breast (Maillot et al. 2009), gastric cancer (Yao et al. 2009) and head and neck cancer (Nurul-Syakima et al. 2011), while it is reduced in gliomas (Ciafrè et al. 2005) and aggressive chronic lymphocytic leukaemia (Marton et al. 2008). The possible explanation is that miR-181 target different genes, depending on the cellular environment. It was also reported that the expression of miR-181a levels correlated with the aggressiveness of the disease in patients with cytogenetically normal acute myeloid leukaemia. Single microRNA miR-181a could be a prognostic factor in AML patients (Schwind et al. 2010; Marcucci et al. 2011). In the present study, we found that the expression of miR-181a was decreased in Ara-C-resistant leukaemia cell lines. In addition, overexpression of miR-181a sensitizes HL-60/Ara-C cells. These results indicated that miR-181a plays a crucial role in Ara-C-resistant phenotype in AML cells.

To date, several targets of miR-181a have been reported including K-ras (Shin et al. 2011), Bcl-2 (Neilson et al. 2007), Prox1 (Kazenwadel et al. 2010), TCRα (Neilson et al. 2007) and hox A11 (Naguibneva et al. 2006). In line with previous findings (Neilson et al. 2007), we found that Bcl-2 is a direct target of miR-181a. Bcl-2 is an important member of a protein family composed of regulators of programmed cell death in both normal and abnormal cells (Youle and Strasser 2008; Reed 1998). It is a pro-survival protein that regulates apoptosis by preventing the release of pro-apoptotic factors from mitochondria (e.g. cytochrome C) and subsequent activation of a caspase cascade (Ola et al. 2011). Bcl-2 is upregulated in many tumours where it promotes survival and chemoresistance. Many efforts are being made to develop anticancer drugs that efficiently inhibit Bcl-2. Previous studies have shown that changes in Bcl-2 family are found in several chemotherapy resistance cell line (Colié et al. 2009; Doudican et al. 2008; Konishi et al. 2006). Studies have shown that during Ara-C-based induction chemotherapy in AML patients, Bcl-2 protein levels increased significantly (Andreeff et al. 1999). Upregulation of Bcl-2 in AML patients results in poor response to chemotherapy (Campos et al. 1993). It has also been reported that knockdown of Bcl-2 induces apoptosis and increases the sensitivity of AML patients blasts to Ara-C (Keith et al. 1995). In this study, we found that Bcl-2

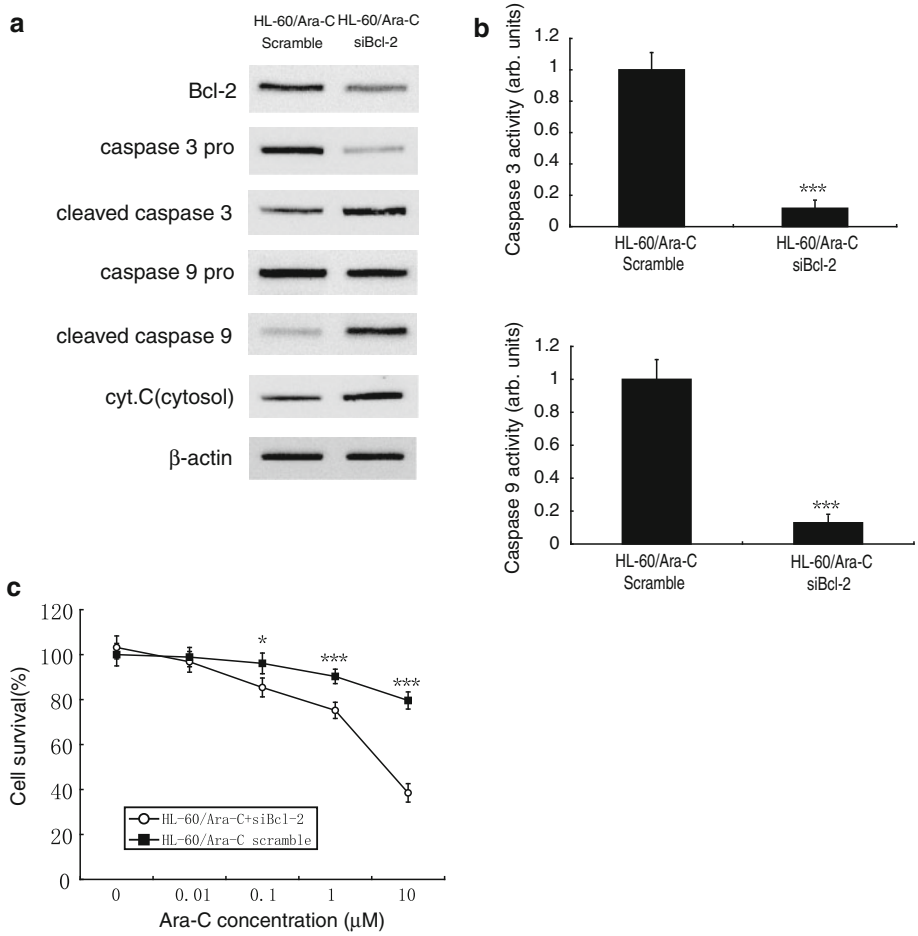

Fig. 5 Bcl-2 plays a crucial role in Ara-C-induced resistance in the HL60 cells. **a** Representative western blots showing Bcl-2, caspase 3, caspase 9 and cytosolic cytochrome protein levels in HL60/Ara-C cells with or without transfection of Bcl-2 siRNA. **b** Activity of caspase 3 and caspase 9 was decreased in HL60/Ara-C cells after transfection of Bcl-2 siRNA compared to scramble controls. Data are mean ± SEM

expression level was increased in HL-60/Ara-C cells and overexpression of miR-181a induced a significant reduction in Bcl-2 mRNA as well as Bcl-2 protein. More importantly, Bcl-2 siRNA mimicked the effect of miR-181a overexpression that reverses the resistant phenotype of HL-60/Ara-C cells, suggesting that downregulation of miR-181a may be involved in Ara-C resistant by upregulation of Bcl-2. To our knowledge, this is the first time anyone linked miR-181a, Bcl-2 expression and Ara-C resistance in HL-60 cells.

In this study, we found that overexpression of miR-181a in HL-60 cells cannot sensitize the cells to Ara-C treatment. It may be that the apoptosis pathway regulation depends on the basal levels of Bcl-2 and miR-181a.

In conclusion, this report has shown that the resistance of Ara-C relates to the downregulation of miR-181 and in turn upregulation of its target gene Bcl-2. The higher expression of Bcl-2 is assumed to inactivate caspase pathway and block apoptosis. Therefore, dysregulation of miR-181a might lead to the acquisition of Ara-C resistance in AML. Targeted treatments that increase endogenous levels of miR-181a might represent novel therapeutic strategies to overcome AML drug resistance.

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Conflict of interest We declare that we have no conflict of interest.

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