Involvement of miR-21 in resistance to daunorubicin by regulating PTEN expression in the leukaemia K562 cell line

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Article info
Article history:
Received 4 October 2010
Revised 17 December 2010
Accepted 20 December 2010
Available online 25 December 2010

Abstract
Recent studies have shown microRNA-21 (miR-21) is overexpressed in several types of cancer and contributes to tumor resistance to chemotherapy. In this study, we investigated whether miR-21 mediated resistance of the leukaemia cell line K562 to the chemotherapeutic agent daunorubicin (DNR). miR-21 expression was upregulated in the DNR resistant cell line K562/DNR compared to its parental line K562. Stable transfection of miR-21 induced drug resistance in K562, while suppression of miR-21 in K562/DNR led to enhanced DNR cytotoxicity. Additional experiments indicate that the mechanism of miR-21 drug resistance involves the PI3K/Akt pathway and changes following PTEN protein expression. This study provides a novel mechanism for understanding leukaemia drug resistance.

1. Introduction
Daunorubicin (DNR) is a chemotherapeutic that belongs to the anthracycline family and is used to treat cancer [1,2]. Since it was developed as a clinical application for treating leukaemia, daunorubicin-based chemotherapy has become one of the most effective anti-cancer drugs [3,4]. However, as with other chemotherapy agents, drug resistance to DNR continues to be a major clinical obstacle for the successful treatment of leukaemia, thus limiting its clinical use [5]. Drug resistance is considered to be a multifactorial phenomenon that involves several major mechanisms, such as increased DNA damage repair, reduced apoptosis, changes in drug metabolism, and increased energy-dependent drug efflux, as well as changes in glutathione transferase and topoisomerase II expression [6,7]. Unfortunately, the specific mechanisms involved in leukaemia DNR drug resistance remain largely unknown.

MicroRNAs (miRNAs) are small, non-coding RNAs that negatively regulate the expression of target genes post-transcriptionally by binding to the 3’UTR of mRNA [8]. Dysregulation of microRNAs has been reported in a variety of human cancers [9]. Increasing evidence indicates that microRNAs play a role in tumor cell resistance and/or sensitivity to chemotherapeutic agents [10]. In particular, miR-21 has been shown to function in the oncogenesis and drug resistance [11], however the role of this microRNA in the leukaemia DNR resistance has not been described. In this work, we show that miR-21 is highly expressed in DNR resistant leukaemia K562 cell line. Additional experiments suggest miR-21 contributes resistance to DNR by regulating PTEN expression in K562 cells. Our findings indicate that upregulation of miR-21 and subsequent downregulation of PTEN is a novel mechanism for DNR resistance in leukaemia.

2. Materials and methods
2.1. Cell culture

The K562 cell line and the K562/DNR-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% fetal 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% fetal bovine serum, 100 g/ml l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin.

2.2. Retroviral transduction of miR-21 and transfection of miR-21 ASO

A 300 bp DNA fragment containing the miR-21 precursor was PCR amplified from human genomic DNA (Roche Applied science).
with the following primers: forward: 5’- AGATCTCATCTTAACAGG CCAGAAATGC-3’ and reverse: 5’- CTGAGCCCCACAGAAGGACCC AGAGT-3’; the Xhol and BglII recognition sites are underlined. The amplicon was cloned into a modified MSCV-PGK-GFP vector (Clontech). To generate a cell line that stably expressed miR-21 (K562-MSCV miR-21), 10 μg expression vector or empty vector with 10 μg packing plasmid (gag/pol and VSVG) was incubated with Fu gene 6 (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 retronectin (Takara); after 24 h, the cells were centrifuged at 1500g for 30 min. The GFP positive cells were approximately 50–70% and sorted by flow cytometry. Control oligonucleotides and miR-21 complementary oligonucleotides were purchased from Invitrogen (Carlsbad, CA). The sequences were as follows: miR-21 antisense oligonucleotide (ASO), 5’-TCAACATCAGCTGATAAGCTA-3’ and control ASO, 5’-GTGATATGTTGACATTCA-3’. The cells were transfected with Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). The oligonucleotides were used at a final concentration of 200 nM in antibiotic-free Opti-MEM medium (Invitrogen). All of the assays were performed 48 h after transfection.

2.3. 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 DNR. Approximately 72 h after DNR 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 with a microplate spectrophotometer. Each experiment was carried out in triplicate and repeated three times.

2.4. MicroRNA array

Total RNA samples were analyzed by CapitalBio (Beijing, China) for miRNA microarray experiments. The chips contain 743 probes in triplicate, corresponding to 576 human (including 122 predicted miRNAs), 358 mouse, and 238 rat miRNAs (based on Sanger miRNAs database miRBase). Experimental procedures were performed as described in detail on the website of CapitalBio http://www.capitalbio.com. Briefly, total RNA was isolated using the miRNasy kit (Qiagen). MicroRNA labeling, hybridization and washing were carried out following manufacturer’s instructions. Double-channel laser scanner LuxScan 10 K/A (CapitalBio) was used for fluorescence scanning, and the image signals were transferred to digital signals using image analysis software LuxScan 3.0 (CapitalBio). Differentially expressed miRNAs were identified by the Significance Analysis of Microarrays (SAM, version 3.0, from Stanford University).

2.5. Real-time PCR

The total cellular RNA was isolated with a RecoverAll kit (Ambion Inc., Austin, Texas) for both the miR-21 and PTEN-mRNA analyses according to the manufacturer’s instructions. To detect miR-21 expression, a TaqMan microRNA Assay (Applied Biosystems, Foster City, CA) was performed as described [12] on an Applied Biosystems ABI 7500 Real Time PCR system. U6 was used as a control. To detect PTEN-mRNA expression, real-time PCR was performed with a Quantitect SYBR Green PCR Kit (Qiagen). β-Actin was used to normalize the PTEN-mRNA expression level. The forward and reverse primers were 5’-CAGAGCCTCTGCTTGG-3’ and 5’-GTGCCCATAGATT-3’, respectively. All of the real-time PCR assays were performed in triplicate.

2.6. Northern blot analysis

Northern blots were performed as described previously [13]. Briefly, 15 μg of total RNA was separated on a 15% denaturing polyacrylamide gel, transferred to GeneScreen Plus membranes (PerkinElmer, Waltham, Mass). The membranes were hybridized at 37 °C for 24 h to γ-32P labelled probe. The probe was end-labelled with [γ-32P]ATP (6000 Ci/mmol) and T4 Polynucleotide Kinase. The miR-21 probe sequence was 5’-TCAACATCAGCTGATAAGCTA-3’, and the U6 probe sequence was 5’-GCAGGGCCCACCTGATCAAAC-3’.

2.7. Luciferase assay

A PTEN 3’ UTR Luciferase reporter was constructed by inserting full length human PTEN 3’ UTR into the XhoI and NotI sites in the psiCHECK2 vector (Promega) downstream from the renilla luciferase coding sequence. The PTEN 3’UTR was PCR amplified from human genomic DNA with the following primers: forward: 5’-TGGCTGATGATTTTTTTTATAGGACGAGG-3’ and reverse, 5’-TCGCAGGGCGCGCAAAATGACTATTCTGATC-3’. Mutations within the putative miR-21 binding sites were created with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutagenic primers were forward, 5’-ACTTTGGCGCAAACGTTTTACCTGAGT-3’ and reverse, 5’-CAACTGCAAAGTAAACTGTTGCCAGTAACCTGATC-3’. The K562 cells were plated in 96-well plates at 5000 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-21 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 Tecxan Spectrafluor Plus machine. The renilla luciferase readings were normalized to the firefly luciferase activity in the corresponding well.

2.8. Western blot

The cells were washed twice with PBS that contained 1 mM phenyl-methylsulphonyl fluoride and then lysed in Lysis Reagent (Sigma–Aldrich, St. Louis, MO). Cell lysates (approximately 30 μg of protein) were loaded on an 8% SDS–PAGE gel and subsequently transferred to a polyvinylidene difluoride membrane (PVDF; Bio-rad). 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 PTEN, p-Akt, and Akt were purchased from Cell Signaling Technology, the β-actin antibody was purchased from Sigma and the PD-DC4 antibody was purchased from Abcam Ltd. Band detection via enzyme-linked chemiluminescence was performed according to the manufacturer’s protocol (ECL; Pierce Biotechnology Inc., Rockford, IL). The protein bands were quantified using ImageJ 1.33 software (NIH), and the data were normalized to β-actin.

2.9. siRNA transfection

A SignalSilence® PTEN 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 70%.
2.10. 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 two groups. A $P < 0.05$ was considered to be significant.

3. Results

3.1. miR-21 is upregulated in the DNR-resistant K562/DNR cell line

Our primary miRNA profiling studies indicated that 3 microRNAs were upregulated (miR-21, miR-125b, and miR-99a) and 9 microRNAs were downregulated (miR-23a, miR-29a, miR-29c, miR-30b, miR-30c, miR-183, miR-221, miR-222, and miR-224) by at least 1.5 fold in the K562/DNR-resistant cells compared to the parental K562 cells. We selected miR-21 to further validate the array results because previous studies have shown that miR-21 is an oncogene that is related to drug resistance [10,11]. Therefore, we first verified differences in miR-21 expression by northern blot (Fig. 1A) and real-time PCR analyses (Fig. 1B). The quantitative PCR results showed that the miR-21 expression level was higher in the K562/DNR cells than in the K562 cells (Fig. 1A, ***$P < 0.001$), indicating that miR-21 may be associated with DNR resistance in K562 cells.

3.2. Overexpression of miR-21 renders the K562 cells resistant to DNR

To directly test the relationship between miR-21 and chemoresistance in the K562 cells, we further studied the DNR-resistant K562 cell line. The survival curves of the K562/DNR cells and the parental K562 cell line are shown in Fig. 2A. The K562 cells were stably transduced with an MSCV-miR-21 vector or empty vector and treated with various doses of DNR. Overexpression of miR-21 was associated with significantly increased survival of the K562 cells (Fig. 2B). At DNR concentrations higher than 0.01 μM, the K562 cells that were transduced with miR-21 had a significantly higher survival than the control group (***$P < 0.005$). This result suggests that miR-21 contributes to DNR resistance in the K562 cells.

3.3. Knockdown of miR-21 partially sensitizes the K562/DNR cells to DNR

We further investigated the effects of miR-21 on DNR-induced cytotoxicity in K562/DNR cells that were transfected with a miR-21 ASO. The cells were transfected with either the miR-21 ASO or a control ASO and were subsequently incubated with various doses of DNR. As is shown in Fig. 2C, the miR-21 ASO effectively reduced the expression of miR-21 (***$P < 0.001$). At DNR concentrations greater than 0.1 μM, K562 cells that were transfected with the miR-21 ASO had a significantly lower survival than the control group (Fig. 2D, ***$P < 0.005$), suggesting that decreasing miR-21 expression alters DNR sensitivity in K562/DNR cells.

3.4. Overexpression of miR-21 activates the PI3K/Akt pathway by downregulating PTEN

Although the PTEN 3′UTR is not a perfect match to the seed sequence of miR-21 because there was a G:U pair (Fig. 3A), a previous study showed that PTEN is a miR-21 target in cholangiocarcinoma cell lines [14]. We thus hypothesized that PTEN is a direct miR-21 target in K562 cells. To confirm this, the K562 cells were co-transfected with PTEN-3′UTR luciferase reporter and miR-21. Transfections with control vector were performed in parallel. As is shown in Fig. 3B, miR-21 markedly decreased the activity of the PTEN-UTR reporter (**$P < 0.01$). However, transfection of the PTEN-3′UTR mutant did not affect reporter activity (#$P > 0.05$). We also assessed whether miR-21 regulated PTEN expression in K562 cells. Overexpression of miR-21 decreased the PTEN protein level (**$P < 0.001$) in K562 cells, whereas knockdown of miR-21 by ASO–miR-21 increased the PTEN protein level (**$P < 0.001$) in K562/DNR cells (Fig. 3C and E); however, no changes were seen in PTEN mRNA expression (Fig. 3D, #$P > 0.05$). Because PTEN is a negative regulator of the PI3K/Akt pathway, we hypothesized that overexpression of miR-21 may activate PI3K/Akt by reducing the PTEN protein level, resulting in K562 resistance to DNR. As is shown in Fig. 3F, K562 cells that were transduced with miR-21 have increased Akt phosphorylation (**$P < 0.001$); additionally, knockdown of miR-21 in the K562/DNR resistant cell line decreased Akt phosphorylation (**$P < 0.001$). These results show that PTEN is a target of miR-21, and that overexpression of miR-21 activates the PI3K/Akt pathway by reducing the PTEN protein level.

Recent studies have also shown that anti-miR-21 treatment sensitises K562 cells to arsenic trioxide by suppressing PDCD4 expression [15–17]. To explore the role of PDCD4 in DNR-induced resistance in K562 cells, we examined PDCD4 protein levels in K562 and K562/DNR cells. Overexpression of miR-21 in the K562 cells or knockdown of miR-21 in the K562/DNR cells did not change the PDCD4 protein levels (#$P > 0.05$), suggesting that PDCD4 is not involved in DNR drug resistance in this cell line.

3.5. PTEN is a key signal molecule in induced K562 DNR resistance

Previous studies have shown that PTEN is involved in drug resistance in several types of cancer [18,19]; however, its role in DNR sensitivity in the K562 cell line remains unclear. To explore the relationship between PTEN and DNR-induced cytotoxicity, we
transfected PTEN siRNA or a scrambled siRNA into K562 cells, followed by treatment with various doses of DNR. PTEN siRNA effectively reduced the PTEN protein level (Fig. 4A). Furthermore, K562 cells that were pre-treated with PTEN siRNA had increased survival compared to the control group (Fig. 4C). More importantly, the K562 cells that were treated with PTEN siRNA had increased pAkt levels (Fig. 4B) and a survival pattern that is similar to cells with miR-21 overexpression, suggesting that miR-21 confers DNR resistance via regulating PTEN in the K562 cells.

4. Discussion

In this paper, for the first time, we show that DNR-induced drug resistance is associated with upregulation of miR-21 in the leukaemia cell line K562. miR-21 may regulate the survival of leukaemia cell lines by targeting PTEN expression and causing subsequent changes in the PI3K/Akt pathway.

Several studies have shown that miR-21 is an oncogenic miRNA that is overexpressed in various types of cancer, such as breast [20], pancreatic endocrine [21], pre-B-cell lymphoma [22], glioblastoma [23] and prostate cancer [24]. Recent studies have indicated that miR-21 contributes to drug resistance in solid tumours through several pathways [21,25–27]. In the present study, we found that miR-21 was upregulated in K562/DNR cells compared to K562 cells, indicating that miR-21 is involved in leukaemia DNR drug resistance. Additional studies involving over and under-expression of miR-21 were performed to confirm the effect of miR-21 on DNR resistance in K562 cells. Our studies show that overexpression of miR-21 attenuates cell death, whereas knockdown of miR-21 promotes cell death.

Numerous miR-21 targets have been identified, including PDCD4 [28], MARCKS [29], LRRFIP1 [27], TGFBR2 [30], and TPM1 [31]. Meng et al. [14,32] first verified that PTEN is a direct target of miR-21 and showed that this microRNA plays a role in human cholangiocarcinoma cell lines and human hepatocellular cancer. Of these miR-21 targets, we focused on the phosphatase and tensin homolog deleted on chromosome 10 (PTEN), because it is an important gene that is involved in leukaemia pathology [33,34] and drug resistance [35,36]. Studies have shown that transfection of PTEN in the drug-resistant AML cell line (HL60AR) or ALL cell line (EU21) causes chemosensitivity to anti-cancer agents [35,36]. Consistent with this finding, we demonstrate here that PTEN is a target of miR-21 and that it plays a role in DNR resistance in the K562 cell line. Moreover, knockdown of PTEN significantly increased cell survival and had an overall effect that was similar to miR-21 overexpression. To our knowledge, this is the first time anyone has described an association between miR-21, PTEN expression and drug resistance in DNR-treated K562 cells.

Fig. 2. miR-21 confers DNR resistance in K562 cells. (A) A survival curve of drug-resistant K562/DNR cells and K562 parental cells. Cell viability was determined using an MTT assay. (B) The K562 cells were transduced with an MSCV-miR-21 vector or an empty vector and were subsequently treated with various doses of DNR. Cell viability was determined using an MTT assay. The error bar shows the S.E. for three independent experiments. (C) K562/DNR cells were transfected with ASO-miR-21 or a control ASO. miR-21 expression was quantified by real-time PCR. (D) K562/DNR cells were transfected with ASO-miR-21 vector, a control ASO, or vehicle and were subsequently treated with various doses of DNR. Cell viability was determined using an MTT assay. The error bar shows the S.E. for three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001.
Moreover, PTEN is a negative regulator of the PI3K/Akt pathway. Consequently, we saw that overexpression of miR-21 or knockdown of PTEN activated Akt kinase. Akt regulates cell growth and inhibits apoptosis via controlling downstream proteins [34].

Fig. 3. miR-21 activates the PI3K/Akt pathway by decreasing PTEN protein levels. (A) A nucleotide comparison between the miR-21 seed sequence and the PTEN 3’UTR sequence. Three nucleotides in the miR-21 seed region were mutated with PCR site-directed mutagenesis; the arrows show the mutated nucleotides. (B) The effect of miR-21 on PTEN was assessed with the luciferase reporter system. The miR-21 expression vector, together with the luciferase reporter vector or control vector, was cotransfected into the K562 cells. (C) Representative western blots showing PTEN, pAkt, and Akt protein levels after transfection with an empty vector or the MSCV-miR-21 vector in K562 cells, as well as the ASO control or ASO-miR-21 in K562/DNR cells. β-Actin was used as a loading control. (D) PTEN mRNA expression was detected by real-time PCR. (E) The graph shows the PTEN protein levels after transfection with miR-21 or ASO-miR-21. (F) The pAkt/Akt protein levels after transfection with miR-21 or ASO-miR-21. (G) Representative western blots showing expression of PDCD4 after transfection with a control vector or the MSCV-miR-21 vector in K562 cells, as well as the ASO control or ASO-miR-21 in K562/DNR cells. β-Actin was used as a loading control. (H) The PDCD4 protein levels after transfection with miR-21 or ASO-miR-21. The error bar indicates the S.E. **P < 0.01, ***P < 0.001, and *P > 0.05.
miR-21 expression and decreased PTEN protein expression in K562 cells. This discrepancy suggests that miR-21 may regulate the chemosensitivity of K562 cells to arsenic trioxide through high-dose daunorubicin in older patients with acute myeloid leukemia. N. Engl. J. Med. 361, 1235–1248.


[9] Cell viability (%)

[10] Cell viability was determined using an MTT assay. The error bars indicate the S.E. *P < 0.05, **P < 0.01, and ***P < 0.001.

Several lines of evidence have shown that Akt phosphorylates GSK-3β [37], Foxo3a [38], mTOR [39] and Bcl-2 [40] in K562 cells and thus regulates cell growth. Further experiments are required to validate the direct downstream targets of Akt that are responsible for this mechanism of drug resistance.

In this study, we found that a miR-21 ASO cannot completely sensitize the K562/DNR cells. This suggests that upregulation of miR-21 is not the only mechanism responsible for DNR resistance. It may be that other microRNAs targeting PTEN are involved in DNR resistance in the leukaemia cell line. Recent studies revealed that PTEN is regulated by multiple microRNAs including miR-17-92 cluster [41], miR-26a [42], miR-216a/217 cluster [43], miR-214 [44], miR-221/222 [45], miR-486 [46] and miR-22 [47]. As is shown in our microRNA array results, miR-221 and miR-222 are differentially expressed in K562 and K562/DNR cells; these microRNAs may contribute to DNR resistance through targeting PTEN. In addition, microRNAs may target other apoptosis related proteins and involve in DNR resistance. miR-125b, which was upregulated in K562/DNR cells, has been shown to suppress the pro-apoptotic protein BAK1, thus inhibiting apoptosis [48]. Another study suggested that miR-221 and miR-222 down regulate c-kit and inhibit erythroleukaemic cell growth [49]. Future studies are needed to assess the role of these microRNAs in DNR resistance.

Previous studies have shown that a miR-21 ASO can modulate the chemosensitivity of K562 cells to arsenic trioxide through downregulation of PDCD4 protein expression [15–17]. However, our data did not indicate that PDCD4 plays a role in DNR resistance in K562 cells. This discrepancy suggests that miR-21 may regulate different targets in response to different drugs. Additionally, the miR-21 expression level may differ in these cell lines.

In summary, our results show that leukaemia cells with elevated miR-21 expression and decreased PTEN protein expression were more resistant to DNR than the control cells. These results may help with the development of personalized treatment for patients who have abnormal levels of miR-21 or PTEN. Furthermore, this novel miR-21/PTEN/P3K/Akt signalling pathway in leukaemia cells may provide drug targets for the sensitisation of tumour cells and could be applied to treat chemotherapy resistance in leukaemia patients. Future investigations using patient samples are needed to further support the function of miR-21 in leukaemia DNR resistance.

Acknowledgments

We thank our laboratory members for helpful discussions and critical reading of the manuscript. This work was funded by the National Natural Science Foundation of China (No. 81070135).

