Original Paper

MicroRNAs Contribute to Promyelocyte Apoptosis in As₂O₃-Treated APL Cells

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Key Words

As₂O₃ • Acute promyelocytic leukemia • Apoptosis • microRNA • PML

Abstract

Background: Arsenic trioxide (As₂O₃), an ancient drug used in traditional Chinese medicine, has substantial anticancer activities, especially in the treatment of patients suffering from acute promyelocytic leukemia (APL); however the underlying mechanisms are not well understood. **Methods:** MTT assay was used to detect the cell viability. Flow Cytometry analysis and caspase-3 activity assay were used to measure apoptosis of APL cells. Caspase-3 and Bax levels were analyzed by western blot and let-7d and miR-766 levels were determined by real-time RT-PCR. **Results:** As₂O₃ significantly inhibited cell viability and induced apoptosis in APL cells. Several microRNAs, including let-7d and miR-766, were dysregulated in APL cells treated with As₂O₃. The expression of caspase-3 and Bax, which are targets of let-7d and miR-766, respectively, were up-regulated in As₂O₃ treated cells. Transfection of let-7d and miR-766 into NB4 cells decreased the expression of caspase-3 and Bax, respectively. Correspondingly, transfection of these microRNAs increased NB4 cell viability. As₂O₃ induced degradation of promyelocytic leukemia (PML), and then induced the down-regulation of both let-7d and miR-766 in NB4 cells. **Conclusions:** We construct a dysregulated microRNA network involved in As₂O₃-induced apoptosis in APL. Targeting this network may be a new strategy for the prevention of side effects associated with APL treatment with As₂O₃.

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Introduction

Acute promyelocytic leukemia (APL), M3 subtype of acute myeloid leukemia (AML), is characterized by a reciprocal translocation between chromosomes 15 and 17, which results in the fusion protein production between the promyelocytic leukemia (PML) gene and the retinoic acid receptor alpha (RAR α) gene, and subsequent inhibition of myeloid cell apoptosis and differentiation [1, 2]. APL accounts for approximately 10-15% of all AML cases and represents a highly malignant form of leukemia with high bleeding tendency and a fatal course of only few weeks [3, 4]. Although two-thirds of APL patients achieved complete remission after the conventional chemotherapy, such as anthracyclines and cytosine arabinoside, high frequency of early death mainly due to the exacerbation of bleeding syndrome and low 5-year disease-free survival rates dwarf them to new drugs [5].

Early in 1971, researchers at the Harbin Medical University in China made the groundbreaking finding that arsenic trioxide (As₂O₃), the main component of arsenic, has prominent efficacy in the treatment of APL [6, 7]. Since then, As₂O₃ has drawn great attention because of its superior therapeutic effects on APL and has been applied on many other tumors, such as prostate cancer, breast cancer, lung cancer, liver cancer and so on [8-10]. Clinical experiments have shown the effectiveness of As₂O₃ in inducing complete clinical remission in patients with newly diagnosed and relapsed cases of APL. The molecular mechanism of anti-leukemic effect of As₂O₃ is complex. Accumulating evidence has suggested that the therapeutic effect of As₂O₃ mainly results from inducing apoptosis of cancer cells, but the underlying mechanisms are not well understood [11].

MicroRNAs (miRNAs) are a class of endogenous small non-coding RNAs, which are 18-22 nt in length. Mature miRNAs bind to the 3'-UTR of target genes and repress translation of target genes or induce degradation of target mRNA [12]. Increasing evidence supports that miRNAs play indispensable roles in many biological processes involved in the pathogenesis of cancer, such as cell proliferation, apoptosis, angiogenesis, inflammation and so on [13-16]. For example, miR-21 has been proved as an oncomiR, which targets multiple key players in cell proliferation and apoptosis. MiR-125b functions as a tumor suppressor in liver cancer and bladder cancer by inducing the abnormal cell proliferation [17-20].

It has been confirmed that some miRNAs, such as miR-125b and miR143/145, contribute to the pathogenesis of APL and the development of drug resistance [21, 22]. Other miRNAs, such as anti-miR-21 and miR-15a/16-1, were reported to enhance the sensitivity of leukemic cells to the anti-cancer effect of As₂O₃ and all-trans retinoic acid [23-25]. However, little attention has been paid to the role of miRNAs in the process of apoptosis induced by As₂O₃ in APL patients. The aim of this study was to investigate the role of miRNAs in the process of apoptosis induced by As₂O₃ in APL cells. We expect to shed new light on the potential mechanisms of As₂O₃ on the treatment of APL and provide new molecular targets for the prevention of As₂O₃-induced side effects.

Materials and Methods

Cell Culture and Treatment

The NB4 and HL-60 cell lines, derived from the marrow of a patient with APL in relapse [26], was cultured in RPMI-1640 medium (Hyclone) supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 U per ml penicillin G and 100 U per ml streptomycin at 37°C in 5% CO₂±95% air. The cells treated with As₂O₃ were collected at 24 h and 48 h for further measurements.

Transfection

Before transfection, NB4 or HL-60 cells were grown in 25 cm² cell culture flasks with 4 ml medium. The miRNA and lipofectamine 2000 (Invitrogen, Carlsbad, CA) were separately mixed with 500 μ l of Opti-MEM® I Reduced Serum Medium (Gibco, Grand Island, NY) for 5 min. Then, the two mixtures were combined and incubated for 20 min at room temperature (RT). The lipofectamine:miRNAs mixture was added to the cells and incubated at 37°C for 36 h for further experiments.

MTT Cell Viability Assay

NB4 or HL-60 cells were seeded in 96-well culture plates with 1×10^4 cells/well, and incubated at 37°C with 5% CO₂. After treated with different concentrations of As₂O₃ or microRNAs, MTT assay (Amresco, Solon, USA) was performed. Briefly, 20 µl of MTT solution (5 mg/ml) was added to each well, and the cells were continuously incubated for 4 h. Formazan crystals were then dissolved in 150 µl DMSO. The optical density (OD) of the wells was measured with a microplate reader (BioTek, Richmond, USA) at 490 nm.

Caspase-3 Activity Assay

To assess the activity of caspase-3, NB4 or HL-60 cells were centrifuged at 600g for 5 min and then lysed in 50 µl of ice-cold cell lysis buffer for 30 min. The lysates were centrifuged at 16,000g for 15 min at 4°C. The supernatant was used for subsequent assay. The fluorogenic substrates for caspase-3 were labeled with the *p*-nitroaniline (*p*NA), which is released from acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-*p*NA), a substrate upon cleavage by caspase-3. The enzyme activity was determined by monitoring the fluorescence produced by free *p*NA using a spectrofluorophotometer (SHIMADZU Corporation, RF-5301PC, Kyoto, Japan) at 405 nm. Caspase-3 activity was expressed in micromole *p*NA liberated as per minute per microgram of protein [27].

Flow Cytometry Analysis

Apoptosis was identified by flow cytometry (BD FACSVantage SE, USA) with an apoptosis detection kit (Beijing Biosea Biotechnology Co., LTD, Beijing, China). NB4 or HL-60 cells containing about 5×10^5 cells were centrifuged with 1000g for 10 min at 4°C and resuspended in binding buffer solution. Then, NB4 or HL-60 cells were double-stained with fluorescein isothiocyanate-labeled-Annexin V (Annexin V-FITC) and propidium iodide (PI). Annexin V-FITC is an indicator for early apoptosis, and PI is a viability dye that stains late apoptotic or necrotic cells.

Western Blotting

For western blot analysis, total protein samples were extracted from NB4 or HL-60 cells by the procedure as previously described [28]. Cells were lysed with RIPA lysis buffer (Beyotime, Jiangsu, China). Sixty µg proteins were fractionated on a 15% SDS-polyacrylamide gel. After electrophoretically transferring to a Pure Nitrocellulose Blotting membrane (Pall Life Science), the blots were probed with primary antibodies. Rabbit polyclonal antibody for caspase-3 and Bax were purchased from Santa Cruz Biotechnology. Anti-GAPDH antibody (Kangchen, Shanghai, China) was used as an internal control. The immunoreactivity was detected using Odyssey Infrared Imaging System (Gene Company Limited, Hongkong, China) and analyzed using Odyssey software (Infrared Imaging System LI-COR Biosciences). The bands were quantified by measuring the band intensity for each group [29].

Real-Time RT-PCR

Total RNA samples were extracted from NB4 or HL-60 cells using Trizol. Let-7d and miR-766 levels were quantified by the mirVana qRT-PCR miRNA Detection Kit (Ambion) in conjunction with Real-time PCR with SYBR Green I, as described in our previous work [30]. After circle reaction, the threshold cycle (Ct) was determined and relative miRNA levels were calculated based on the Ct values and normalized by U6 level in each sample.

Microarray Analysis

Microarray assay for miRNAs expression profile was performed by the Kangchen (KangChen Bio-tech Inc., Shanghai, China). Three samples including NB4 cells without treatment, NB4 cells treated with 2 µM As₂O₃ for 24 h and 48 h were assayed using the miRNA microarray technology miRCURY™ LNA Array (Exiqon Company, Denmark). The array output was received in Excel spreadsheets as lists of raw data and also as "simple detectable" data, which were the average of 4 signal values for each miRNA on the array. miRNAs with a DiffScore ≤ -10 and ≥ 10, corresponding to a *p*-value of 0.05, were considered as significantly differentially expressed.

Luciferase Reporter Assays

Caspase-3/Bax 3'-UTR containing the conserved let-7d/miR-766 binding sites were synthesized by Invitrogen and amplified by PCR. The synthesized sequence and primers were: synthesized sequence: Caspase-3: CTC ATG CTG CAG AGG GTA CTT TAA GAC ATA CTC CTT CCA TCA AAT AGA ACCA CTA TGA AGC TAC

CTC AAA CTT CCA GTC AGG TAG TTG CAA TTG A; Bax: CCC CGA TTC ATC TAC CCT GCT GAC CTC CCA GTG ACC CCT GAC CTC ACT GTG ACC TTG ACT TGA TTA GTG CCT TCT GCC CTC CCT GGA GCC TCC ACT GCC TCT GGA ATT GCT CA. The synthesized sequences with a mismatch in the seed sequence of the putative let-7d/miR-766 binding site were: Caspase-3: CTC ATG CTG CAG AGG GTA CTT TAA GAC ATA CTC CTT CCA TCA AAT A GTA CCA CTA TGA AGG CTG GAC AAA CTT CCA GTC AGG TAG TTG CAA TTG A. Bax: CCC CGA TTC ATC TAC CCT GCT GAC CTC CCA GTG ACC CCT GAC CTC ACT GTG ACC TTG ACT TGA TTA GTG CCT TCT GCC CTC CGC ACT CCC TCC ACT GCC TCT GGA ATT GCT CA. The PCR fragment was cloned downstream of the luciferase gene between the SacI and HindIII sites in pMIR-Report (Promega). 0.1 µg of the luciferase reporters containing the 3'-UTR were cotransfected with let-7d/miR-766 mimics into HEK-293 cells using lipofectamine 2000 (Invitrogen, Carlsbad, CA). As an internal control, 10 ng of renilla luciferase reporters were also included. 48 h after transfection, the cells were collected and dual luciferase activities were measured by a luminometer according to the manufacturer's instructions.

MiRNA Targets Databases and Apoptosis Pathway

We used miRNA-targets interactions documented in the TargetScan database [31]. The apoptosis pathway was derived from the KEGG database (<http://www.genome.jp/kegg/pathway/hsa/hsa04210.html>).

Statistical Assays

All data are presented as mean±SEM. One way analysis of variance ANOVA followed by Dunnett's post-hoc test was used for multiple comparisons. A two-tailed value of $p < 0.05$ was considered statistically significant difference. Data were analyzed using the GraphPad Prism 5.0 and SPSS 14.0.

Results

Growth Inhibition and Apoptosis of APL Cells Induced by As₂O₃

As₂O₃ is one of the most highly effective agents for APL therapy [32]. It was suggested that the therapeutic plasma concentration for treating APL was 1–2 µM [33]. In this study, we found that As₂O₃ decreased the viability of NB4 and HL-60 cells in a dose- and time-dependent manner using MTT assay. As shown in Fig. 1A, the IC₅₀ of As₂O₃ was 1.673 µM in NB4 cells and 1.857 µM in HL-60 cells after 48h of treatment; thus, 2 µM As₂O₃ was used in the subsequent experiments. To investigate whether the observed reduction of viability was associated with cell apoptosis, flow cytometry assay was applied to detect the apoptotic rate of NB4 and HL-60 cells treated with As₂O₃. As shown in Fig. 1B, compared with the control group, apoptosis was markedly increased in NB4 and HL-60 cells after treatment with 2 µM As₂O₃ for 48h.

Both caspase-dependent and caspase-independent pathways are known to be involved in the process of apoptosis. We investigated whether the apoptosis of APL cells caused by As₂O₃ was associated with the activation of caspase-3. Caspase-3 activity was significantly increased in the NB4 and HL-60 cells treated with As₂O₃ (Fig. 1C), suggesting that the As₂O₃-induced apoptotic cell death is caspase-dependent.

Down-Regulation of Let-7d and MiR-766 Expression by As₂O₃

Our miRNA microarray analysis revealed the differential expressions of miRNAs between the NB4 cells with and without 2 µM As₂O₃ treatment for 48h. Let-7d, miR-128a, and miR-766 etc. were significantly down-regulated in As₂O₃-treated NB4 cells compared with control NB4 cells without As₂O₃ treatment (Fig. 2A). The down-regulation of let-7d and miR-766 were further verified in NB4 and HL-60 cells by qRT-PCR (Fig. 2B). By bioinformatics analysis, we identified several candidate targets for these miRNAs, which based on the KEGG database are relevant to apoptosis such as caspase-3 and Bax. We therefore analyzed their protein levels using western blot. As illustrated in Fig. 2C, caspase-3 and Bax genes contain the putative binding sites for let-7d and miR-766, respectively. Western Blot showed that the expression of caspase-3 and Bax proteins were up-regulated in NB4 and HL-60 cells treated with As₂O₃, which is negatively correlated with the levels of let-7d and miR-766 (Fig. 2D).

Fig. 1. As₂O₃ reduces cell viability and induces apoptosis in APL cells. (A) As₂O₃ decreased the viability of NB4 and HL-60 cells in a time- and dose-dependent manner, as assessed by MTT. (B) As₂O₃ increased the apoptosis of NB4 and HL-60 cells, as assessed by flow cytometry. (C) As₂O₃ enhances caspase-3 activity in NB4 and HL-60 cells. n=6, **p<0.01 vs control.

Fig. 2. Dysregulation of miRNAs induced by As₂O₃ in APL cells. (A) miRNA microarray analysis revealed the differential expression of miRNAs in NB4 cells with As₂O₃ treatment, compared with control group. (B) Validation of differentially expressed miRNAs by real-time PCR. (C) The sequences showed the unique sites of miRNA:mRNA complementary between let-7d and caspase-3, and between miR-766 and Bax for human genes. The Genbank accession numbers of the genes are indicated in the brackets, and the positions of the target sites are numbered. (D) Increased expression of cleaved-caspase-3 and Bax in NB4 and HL-60 cells treated with As₂O₃ compared with control group by Western blot. n=4, **p<0.01 vs control.

Increased expression of cleaved-caspase-3 and Bax in NB4 and HL-60 cells treated with As₂O₃ compared with control group by Western blot. n=4, **p<0.01 vs control.

Fig. 3. Let-7d and miR-766 post-transcriptionally regulate caspase-3 and Bax, respectively. (A and B) Compared with the control, transfection of let-7d/miR-766 with the luciferase reporter gene linked to the wild-type 3'-UTR of caspase-3/Bax resulted in a significant decrease of luciferase activity, whereas NC showed no effects. Compared with the control, transfection of let-7d and miR-766 resulted in significant decreases of caspase-3 (C) and Bax (D) at protein levels, respectively. Co-treatment with AMO-let-7d and AMO-766 alleviated the decreases of caspase-3 and Bax, whereas NC showed no effects. (E and F) Real-time PCR shows that let-7d/miR-766 has no effects on the expression of caspase-3/Bax mRNA. n=3, * $p < 0.05$, ** $p < 0.01$ vs control; # $p < 0.05$, ## $p < 0.01$ vs let-7d or miR-766. AMO-let-7d and AMO-766: let-7d inhibitor and miR-766 inhibitor; NC: negative control.

Let-7d and MiR-766 Directly Regulate the Expression of Caspase-3 and Bax respectively in NB4 cells

To evaluate whether let-7d/miR-766 could interfere with caspase-3/Bax's 3'-UTR, we utilized the pMIR-Reporter containing the binding sites of caspase-3/Bax for let-7d/miR-766. As illustrated in Fig. 3A & 3B, overexpression of let-7d/miR-766 caused a significant reduction of luciferase activities compared with that of negative control, whereas let-7d/miR-766 had no effects on the pMIR-vector containing a mismatch in the seed sequence of the putative binding site. The reduction of luciferase activities were efficiently reversed by their antisense oligomers AMO-let-7d/ AMO-766. Further study showed that the expression of pro-caspase-3 protein was significantly down-regulated in NB4 cells transfected with let-7d, and it returned to a normal level in cells treated with antisense-let-7d. Negative control (NC) failed to do so (Fig. 3C). As depicted in Fig. 3D, Bax expression displayed similar reduction in NB4 cells transfected with miR-766. And, real-time PCR showed that let-7d/miR-766 had no effect on the expression of caspase-3/Bax mRNA (Fig. 3E & 3F). These results indicated that let-7d and miR-766 post-transcriptionally regulate caspase-3 and Bax, respectively.

Degradation of PML Gene Induced by As₂O₃

If the reduction of let-7d and miR-766 mediated the As₂O₃-induced apoptosis in NB4 cells, transfection of let-7d antisense (AMO-let-7d) or miR-766 antisense (AMO-766) alone

Fig. 4. Inhibition of let-7d and miR-766 induced apoptosis in APL cells. AMO-let-7d or AMO-766 significantly reduced cell viability (A), and enhanced caspase-3 activity (B) respectively. n=5, * $p < 0.05$, ** $p < 0.01$ vs control; # $p < 0.05$, ## $p < 0.01$ vs AMO.

Fig. 5. The effect of let-7d/miR-766 on down-regulated cell viability induced by As₂O₃. n=6, ** $p < 0.01$ vs control; # $p < 0.05$ vs As₂O₃.

into NB4 cells should be able to cause apoptosis. Indeed, as illustrated in Fig. 4A, AMO-let-7d or AMO-766 significantly reduced APL cell viability, and co-transfection of let-7d/miR-766 and AMO-let-7d/AMO-766 abolished the reduction of cell viability. Moreover, transfection of AMO-let-7d or AMO-766 into NB4 and HL-60 cells also enhanced caspase-3 activity and the elevation was abolished by their miRNA mimics respectively, which is consistent with the results of cell viability (Fig. 4B).

Fig. 6. Forced overexpression of PML increases the expression of let-7d and miR-766 in NB4 cells. (A) Western Blot showed the down-regulation of PML in the NB4 cells treated with As₂O₃. After transfecting the PML plasmid into NB4 cells, PML was markedly increased both at protein (B) and mRNA (C) levels. (D) Over-expression of PML up-regulated the expression of let-7d and miR-766. n=3, * *p* < 0.05 vs control, ** *p* < 0.01 vs control.

To examine whether over-expression of let-7d or miR-766 was able to decrease cell apoptosis in NB4 cells treated with As₂O₃. We transfected let-7d/miR-766 or both of them into NB4 cells before treated with As₂O₃. As shown in Fig. 5, let-7d or miR-766 alone has no effect on the down-regulated cell viability induced by As₂O₃, whereas co-transfection of let-7d and miR-766 could alleviate the reduction of cell viability by As₂O₃, indicating that As₂O₃ stimulates the expression imbalance of many miRNAs rather than a single miRNA, and then activates the apoptosis pathway.

As₂O₃ was found to exert its therapeutic effect by promoting degradation of PML gene, which could regulate the expression of several miRNAs [34, 35]. We assumed that As₂O₃ might inhibit the expression of let-7d and miR-766 through degradation of PML. We found that NB4 cells treated with 2 μ M As₂O₃ for 48h down-regulated the level of PML (Fig. 6A). After transfecting the PML plasmid into NB4 cells, PML was markedly increased both at protein and mRNA levels (Fig. 6B & 6C). Meanwhile, Fig. 6D showed that PML could increase the level of let-7d and miR-766, but the empty vector failed to do so. These results indicated that As₂O₃ inhibited let-7d and miR-766 by regulating PML gene.

Discussion

As₂O₃ was introduced as a new and efficient treatment alternative for APL patients [36, 37]. miRNAs play important roles in the initiation and progression of APL. Some APL-related miRNAs have been confirmed, such as miR-143/145 and miR-125b [21, 22, 38]. However, the role of miRNAs in the process of As₂O₃-induced apoptosis is not well understood. The present study demonstrated that miRNAs are dysregulated in APL cells treatment with As₂O₃. Our results revealed that 2 μ M As₂O₃, a clinically relevant dosage, induced aberrant expression of several miRNAs including let-7d and miR-766. Overexpression of let-7d or miR-766 significantly inhibited the expression of proapoptotic genes caspase-3 and Bax. Furthermore, transfection of AMO-let-7d or AMO-766 into APL cells reduced cell viability and up-regulated caspase-3 activity.

Fig. 7. Differentially expressed miRNA-target network. Nodes with V sign are differentially expressed miRNAs. Nodes with round rectangle are the target genes. Edges in the network denote the miRNA-target relationship. The nodes marked with pink and yellow color are analyzed in detail in the text.

Some studies found that the degree of target down-regulation by miRNAs is quantitatively modest and even an over-expressed miRNA typically down-regulates most of its endogenous targets by less than 50%, but most proteins should remain effective over this degree of inhibition [39, 40]. Therefore, we speculate that As₂O₃ disturbs the homeostasis of miRNAs and induces alteration of many miRNAs. Our study showed that As₂O₃ induced apoptosis via disrupting multiple miRNAs rather than a single miRNA in NB4 and HL-60 cells. As shown in Figure 7, these dysregulated miRNAs may have cross-talks with their targets and form a connected network involved in the apoptosis process.

Studies have shown that As₂O₃ exerts dual-effects on the differentiation of APL cells in a dose-dependent manner. Low dose (<0.5 μ M) of As₂O₃ facilitates the differentiation of APL cells, which may be mediated by the PKA-cAMP signaling pathway and RAR/RXR signaling pathway to promote histone acetylation. On the contrary, higher dose (>0.5 μ M) of As₂O₃ initiates apoptosis via the degradation of PML/RAR α oncoprotein, disruption of the balance between anti-apoptotic and pro-apoptotic proteins [5], down-regulation of mitochondrial transmembrane potentials, opening of mitochondrial permeability transition pore, and release of cytochrome C and other pro-apoptotic cytokines. In addition, As₂O₃ induced apoptosis through activation of JNK pathway, modulation of ROS production in APL cells, and interference of cell cycle.

The presence of t(15;17) with the PML-RARA fusion gene is considered to be the hallmark of APL, also known as AML type M3 [41]. APL patients with the PML-RARA fusion gene aberration have been shown to be highly sensitive to retinoid differentiating agents, such as all-trans retinoic acid. Zhang et al. found that As₂O₃ directly binds to PML protein and induces its degradation [34]. A following study by Lang et al. [42] showed that As₂O₃ promotes cytoplasmic sequestration of PML and PML/RAR α through the inhibition of PML body recycling. PML/RAR α fusion protein has been confirmed as a transcription factor that

modulates the transcription of many miRNAs [35]. In our study, 2 μ M As₂O₃ induced the degradation of PML in NB4 cells. Thus, we assumed that As₂O₃ reduces the expression of let-7d and miR-766 through the degradation of PML and the inhibition of PML body recycling, which deserves our future detailed research.

Taken together, the present study found that As₂O₃ induced dysregulation of some apoptosis-related miRNAs in APL cells and the miRNA-target interactions formed a network in the process of apoptosis induced by As₂O₃ in APL cells. Moreover, our results indicated that As₂O₃ reduced the expression of let-7d and miR-766 through the degradation of PML and the inhibition of PML body recycling. Our future study will focus on the investigation of the miRNAs that are differentially expressed in patients suffering from APL compared with patients cured with As₂O₃. We expect to develop strategies to prevent side effects associated with As₂O₃ treatment of APL patients using combination of miRNAs and As₂O₃, which warrants our further detailed research.

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Conflict of Interests

The authors state no competing interests.

References

- 1 Saeed S, Logie C, Stunnenberg HG, Martens JH: Genome-wide functions of pml-raralpha in acute promyelocytic leukaemia. *Br J Cancer* 2011;104:554-558.
- 2 Mistry AR, Pedersen EW, Solomon E, Grimwade D: The molecular pathogenesis of acute promyelocytic leukaemia: Implications for the clinical management of the disease. *Blood Rev* 2003;17:71-97.
- 3 Wang ZY, Chen Z: Acute promyelocytic leukemia: From highly fatal to highly curable. *Blood* 2008;111:2505-2515.
- 4 Jing Y: The pml-raralpha fusion protein and targeted therapy for acute promyelocytic leukemia. *Leuk Lymphoma* 2004;45:639-648.
- 5 Shen ZX, Shi ZZ, Fang J, Gu BW, Li JM, Zhu YM, Shi JY, Zheng PZ, Yan H, Liu YF, Chen Y, Shen Y, Wu W, Tang W, Waxman S, De The H, Wang ZY, Chen SJ, Chen Z: All-trans retinoic acid/as2o3 combination yields a high quality remission and survival in newly diagnosed acute promyelocytic leukemia. *Proc Natl Acad Sci U S A* 2004;101:5328-5335.
- 6 Cyranoski D: Arsenic patent keeps drug for rare cancer out of reach of many. *Nat Med* 2007;13:1005.
- 7 Zhang TD LY: Clinical analysis and empirical study on treatment of ailing no. 1 in 62 cases of acute promyelocytic leukemia. *J Integr Tradit West Med (Chin)* 1984;4:2.
- 8 Chiu HW, Chen YA, Ho SY, Wang YJ: Arsenic trioxide enhances the radiation sensitivity of androgen-dependent and -independent human prostate cancer cells. *PloS one* 2012;7:e31579.
- 9 Sun RC, Board PG, Blackburn AC: Targeting metabolism with arsenic trioxide and dichloroacetate in breast cancer cells. *Mol cancer* 2011;10:142.
- 10 Han B, Zhou G, Zhang Q, Zhang J, Wang X, Tang W, Kakudo K: Effect of arsenic trioxide (ato) on human lung carcinoma pg cell line: Ato induced apoptosis of pg cells and decreased expression of bcl-2, pgp. *J Exp Ther Oncol* 2004;4:335-342.

- 11 Lengfelder E, Hofmann WK, Nowak D: Impact of arsenic trioxide in the treatment of acute promyelocytic leukemia. *Leukemia* 2012;26:433-442.
- 12 van Rooij E: The art of microRNA research. *Circ Res* 2011;108:219-234.
- 13 Primo MN, Bak RO, Schibler B, Mikkelsen JG: Regulation of pro-inflammatory cytokines tnfa and il24 by microRNA-203 in primary keratinocytes. *Cytokine* 2012;60:741-748.
- 14 Yu B, Qian T, Wang Y, Zhou S, Ding G, Ding F, Gu X: Mir-182 inhibits schwann cell proliferation and migration by targeting fgf9 and ntm, respectively at an early stage following sciatic nerve injury. *Nucleic Acids Res* 2012; 40:10356-10365.
- 15 Qin Y, Yu Y, Dong H, Bian X, Guo X, Dong S: MicroRNA 21 inhibits left ventricular remodeling in the early phase of rat model with ischemia-reperfusion injury by suppressing cell apoptosis. *Int J Med Sci* 2012;9:413-423.
- 16 Hansen TF, Andersen CL, Nielsen BS, Spindler KL, Sorensen FB, Lindebjerg J, Brandslund I, Jakobsen A: Elevated microRNA-126 is associated with high vascular endothelial growth factor receptor 2 expression levels and high microvessel density in colorectal cancer. *Oncol Lett* 2011;2:1101-1106.
- 17 Liu ZL, Wang H, Liu J, Wang ZX: MicroRNA-21 (mir-21) expression promotes growth, metastasis, and chemo- or radioresistance in non-small cell lung cancer cells by targeting pten. *Mol Cell Biochem* 2013;372:35-45.
- 18 Jia HY, Wang YX, Yan WT, Li HY, Tian YZ, Wang SM, Zhao HL: MicroRNA-125b functions as a tumor suppressor in hepatocellular carcinoma cells. *Int J Mol Sci* 2012;13:8762-8774.
- 19 Huang L, Luo J, Cai Q, Pan Q, Zeng H, Guo Z, Dong W, Huang J, Lin T: MicroRNA-125b suppresses the development of bladder cancer by targeting e2f3. *Int J Cancer* 2011;128:1758-1769.
- 20 Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY: MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. *Cell Res* 2008;18:350-359.
- 21 Batliner J, Buehrer E, Fey MF, Tschan MP: Inhibition of the mir-143/145 cluster attenuated neutrophil differentiation of apl cells. *Leuk Res* 2012;36:237-240.
- 22 Zhang H, Luo XQ, Feng DD, Zhang XJ, Wu J, Zheng YS, Chen X, Xu L, Chen YQ: Upregulation of microRNA-125b contributes to leukemogenesis and increases drug resistance in pediatric acute promyelocytic leukemia. *Mol Cancer* 2011;10:108.
- 23 Gao SM, Yang J, Chen C, Zhang S, Xing CY, Li H, Wu J, Jiang L: Mir-15a/16-1 enhances retinoic acid-mediated differentiation of leukemic cells and is up-regulated by retinoic acid. *Leuk Lymphoma* 2011;52:2365-2371.
- 24 Cao Y, Yu SL, Wang Y, Guo GY, Ding Q, An RH: MicroRNA-dependent regulation of pten after arsenic trioxide treatment in bladder cancer cell line t24. *Tumour Biol* 2011;32:179-188.
- 25 Li Y, Zhu X, Gu J, Dong D, Yao J, Lin C, Huang K, Fei J: Anti-mir-21 oligonucleotide sensitizes leukemic k562 cells to arsenic trioxide by inducing apoptosis. *Cancer Sci* 2010;101:948-954.
- 26 Lanotte M, Martin-Thouvenin V, Najman S, Balerini P, Valensi F, Berger R: Nb4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (m3). *Blood* 1991;77:1080-1086.
- 27 Chu W, Li X, Li C, Wan L, Shi H, Song X, Liu X, Chen X, Zhang C, Shan H, Lu Y, Yang B: Tgfb3, a potential negative regulator of tgfbeta signaling, protects cardiac fibroblasts from hypoxia-induced apoptosis. *J Cell Physiol* 2011;226:2586-2594.
- 28 Zhou J, Hu L, Cui Z, Jiang X, Wang G, Krissansen GW, Sun X: Interaction of sdf-1alpha and cxcr4 plays an important role in pulmonary cellular infiltration in differentiation syndrome. *Int J Hematol* 2010;91:293-302.
- 29 Liang H, Zhang C, Ban T, Liu Y, Mei L, Piao X, Zhao D, Lu Y, Chu W, Yang B: A novel reciprocal loop between microRNA-21 and tgfbeta3 is involved in cardiac fibrosis. *Int J Biochem Cell Biol* 2012;44:2152-2160.
- 30 Yang B, Lin H, Xiao J, Lu Y, Luo X, Li B, Zhang Y, Xu C, Bai Y, Wang H, Chen G, Wang Z: The muscle-specific microRNA mir-1 regulates cardiac arrhythmogenic potential by targeting gja1 and kcnj2. *Nat Med* 2007;13:486-491.
- 31 Gong X, Wu R, Wang H, Guo X, Wang D, Gu Y, Zhang Y, Zhao W, Cheng L, Wang C, Guo Z: Evaluating the consistency of differential expression of microRNA detected in human cancers. *Mol Cancer Ther* 2011;10:752-760.
- 32 Zhou J, Zhang Y, Li J, Li X, Hou J, Zhao Y, Liu X, Han X, Hu L, Wang S, Zhao Y, Zhang Y, Fan S, Lv C, Li L, Zhu L: Single-agent arsenic trioxide in the treatment of children with newly diagnosed acute promyelocytic leukemia. *Blood* 2010;115:1697-1702.

- 33 Shen ZX, Chen GQ, Ni JH, Li XS, Xiong SM, Qiu QY, Zhu J, Tang W, Sun GL, Yang KQ, Chen Y, Zhou L, Fang ZW, Wang YT, Ma J, Zhang P, Zhang TD, Chen SJ, Chen Z, Wang ZY: Use of arsenic trioxide (as₂o₃) in the treatment of acute promyelocytic leukemia (apl): li. Clinical efficacy and pharmacokinetics in relapsed patients. *Blood* 1997;89:3354-3360.
- 34 Zhang XW, Yan XJ, Zhou ZR, Yang FF, Wu ZY, Sun HB, Liang WX, Song AX, Lallemand-Breitenbach V, Jeanne M, Zhang QY, Yang HY, Huang QH, Zhou GB, Tong JH, Zhang Y, Wu JH, Hu HY, de The H, Chen SJ, Chen Z: Arsenic trioxide controls the fate of the pml-raralpha oncoprotein by directly binding pml. *Science* 2010;328:240-243.
- 35 Saumet A, Vetter G, Bouttier M, Portales-Casamar E, Wasserman WW, Maurin T, Mari B, Barbry P, Vallar L, Friederich E, Arar K, Cassinat B, Chomienne C, Lecellier CH: Transcriptional repression of microRNA genes by pml-rara increases expression of key cancer proteins in acute promyelocytic leukemia. *Blood* 2009;113:412-421.
- 36 Breccia M, Lo-Coco F: Arsenic trioxide for management of acute promyelocytic leukemia: Current evidence on its role in front-line therapy and recurrent disease. *Expert Opin Pharmacother* 2012;13:1031-1043.
- 37 Chendamarai E, Balasubramanian P, George B, Viswabandya A, Abraham A, Ahmed R, Alex AA, Ganesan S, Lakshmi KM, Sitaram U, Nair SC, Chandhy M, Janet NB, Srivastava VM, Srivastava A, Mathews V: Role of minimal residual disease monitoring in acute promyelocytic leukemia treated with arsenic trioxide in frontline therapy. *Blood* 2012;119:3413-3419.
- 38 Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM: Mir-15 and mir-16 induce apoptosis by targeting bcl2. *Proc Natl Acad Sci U S A* 2005;102:13944-13949.
- 39 Inui M, Martello G, Piccolo S: MicroRNA control of signal transduction. *Nat Rev Mol Cell Biol* 2010;11:252-263.
- 40 Rizvi AA, McLaughlin N: Impact of comprehensive diabetes specialty care in the management of older adults with stage 3 or 4 nephropathy. *South Med J* 2009;102:985.
- 41 Pandolfi PP, Alcalay M, Fagioli M, Zangrilli D, Mencarelli A, Diverio D, Biondi A, Lo Coco F, Rambaldi A, Grignani F, et al.: Genomic variability and alternative splicing generate multiple pml/rar alpha transcripts that encode aberrant pml proteins and pml/rar alpha isoforms in acute promyelocytic leukaemia. *EMBO J* 1992;11:1397-1407.
- 42 Lang E, Grudic A, Pankiv S, Bruserud O, Simonsen A, Bjerkvig R, Bjoras M, Boe SO: The arsenic-based cure of acute promyelocytic leukemia promotes cytoplasmic sequestration of pml and pml/rara through inhibition of pml body recycling. *Blood* 2012;120:847-857.