

Evaluation of apoptosis induced by exposure to antineoplastic drugs in peripheral blood lymphocytes of nurses

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Abstract. Cytostatic antineoplastic drugs are considered carcinogenic and mutagenic risk factors for health workers who are occupationally exposed to them; however, the molecular mechanisms underlying these effects remain to be elucidated. Therefore, the present study aimed to investigate the underlying mechanisms of antineoplastic drugs-induced apoptosis of peripheral blood lymphocytes (PBLs) obtained from oncology nurses handling antineoplastic drugs. A microRNA (miRNA/miR) polymerase chain reaction (PCR) array was performed to analyze the expression levels of miRNAs in the PBLs from 3 trained nurses occupationally exposed to antineoplastic drugs. The effects of miR-34a on cell proliferation and apoptosis in temozolomide (TMZ) treated PBLs were analyzed by cell counting kit-8 and flow cytometry assays. The protein expression levels of B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein, caspase-3 and caspase-9 were determined by western blot analysis, and miR-34a expression levels were detected using quantitative reverse transcription-PCR. The results of the present study demonstrated that miR-34a was significantly upregulated in oncology nurses that were occupationally exposed to antineoplastic drugs. In addition, TMZ suppressed cell proliferation and induced apoptosis, by promoting the expression of miR-34a, in a dose-dependent manner, and also inhibited the expression of Bcl-2. Furthermore, knockdown of miR-34a was able to reverse the reduction of cell proliferation and promotion of apoptosis induced by TMZ in PBLs. Together, these results indicated that abnormal expression of miR-34a may be considered a diagnostic marker in nurses occupationally exposed to antineoplastic drugs.

Introduction

Chemotherapy is one of the main clinical therapeutic strategies used to treat tumors; however, antitumor drugs not only target tumor cells, but also affect normal cells. Antineoplastic drugs are known to be carcinogenic, teratogenic and mutagenic to humans, which may lead to mutation, cancer and deformity (1,2). Increasing evidence has suggested that the use of antineoplastic drugs has led to anxiety regarding the possible hazards to staff and workers involved in the preparation, administration and production of cytotoxic agents (3-5). However, the underlying molecular mechanisms of antineoplastic drugs-induced cytotoxicity remain unclear.

MicroRNAs (miRNAs/miRs) are a class of endogenous small non-coding RNAs, 19-25 nt in length, which act as post-transcriptional gene regulators by binding to a target site in the 3'-untranslated region of mRNAs (6). It has previously been demonstrated that alterations in miRNAs may be induced in response to environmental agents and pollutants, which may lead to various health-associated problems (7). Previous studies have indicated that some chemicals, including polycyclic aromatic hydrocarbons, aldehydes and endocrine disruptors, are able to alter *in vitro* miRNA expression (8-10). However, whether miRNAs participate in the toxic effects of antineoplastic drugs in nurses that are occupationally exposed to them remains largely unknown.

Glioma, which has an incidence rate of 5 per 100,000 individuals, is one of the most life-threatening malignant diseases (11). Glioma is difficult to resect due to the infiltrative nature of the tumor, and these tumors almost invariably recur, rapidly leading to mortality (12). Surgical resection alone is inadequate; therefore, effective antineoplastic drugs are required. Temozolomide (TMZ) is a novel imidazotetrazine drug, which has demonstrated efficacy for patients with recurrent glioma (13). However, limited studies have focused on the toxic effects and underlying mechanisms of TMZ in nurses occupationally exposed to this drug.

The primary aim of the present study was to elucidate the toxic effects and underlying molecular mechanisms of TMZ-induced apoptosis in nurses. Considering the important roles of miR-34a and apoptosis in glioma (14,15), the present study investigated whether miR-34a may contribute to the toxic effects of TMZ. To the best of our knowledge, the present study is the first to demonstrate that miRNA expression profiles

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may provide molecular biomarkers in nurses occupationally exposed to TMZ.

Materials and methods

Subjects and drugs. The present study recruited 20 female nurses who regularly handled antineoplastic drugs (age 35.3 ± 9.2 years) from The Workers' Hospital of Tangshan City (Tangshan, China) (oncology nurses), and 20 female subjects (age, 33.6 ± 6.2 years) from the administrative department at the same hospital, who had no occupational contact with antineoplastic drugs (control nurses). TMZ was purchased from Hangzhou Hesu Chemical Technology Co., Ltd. (Hangzhou, China).

The present study was approved by the Medical Ethics Committee of the Workers' Hospital of Tangshan City (permit no. CMU6206-3008). The need for written consent was deemed unnecessary and was waived by the Institutional Ethics Review Board of The Workers' Hospital of Tangshan City. Participation was voluntary, and all participants received detailed information concerning the aims of the research.

Sample collection. All subjects recruited to the present study provided a single blood sample. Venous blood samples (~5 ml) were drawn from each subject into heparinized tubes. Samples were coded and processed within 2 h. Subsequently, lymphocytes were isolated and washed.

Cell culture, treatment and transfection. Peripheral blood lymphocytes (PBLs) derived from whole blood of oncology nurses and control nurses by Ficoll gradient centrifugation ($400 \times g$ for 20 min at room temperature) were cultured for 48 h at 37°C in RPMI-1640 (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) medium containing 20% heat-inactivated fetal bovine serum (Hyclone; GE Healthcare Life Sciences), 1% L-glutamine and antibiotics (100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin). For TMZ treatment, different or indicated doses of TMZ were added to overnight-cultured cells for the indicated times.

For transfection experiments, miR-34a mimics (5'-ACC GUCACAGAAUCGACCAACA-3') and inhibitor (5'-ACA ACCAGCUAAGACACUGCCA-3'), alongside their relative negative controls (NC) (5'-UUCUCCGAACGUGUCACG UTT-3'), were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). PBLs were plated in 6-well culture plates at a density of 1×10^5 cells/well and were transfected following 24 h incubation. The miR-34a mimics, mimics control, miR-34a inhibitor and inhibitor control were transfected into the PBLs using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in Opti-MEM medium (Gibco; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Cancer miRNA polymerase chain reaction (PCR) array. The expression profile of 88 cancer-associated miRNAs was determined using a 96-well cancer RT² miRNA PCR array, purchased from SABiosciences (Frederick, MD, USA). Briefly, peripheral blood samples were randomly obtained from 3 oncology nurses and 3 control nurses (all nurses mentioned in the 'subjects and drugs' subsection).

Total RNA from PBLs was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and was reverse transcribed using the RT² miRNA First Strand kit (SABiosciences) according to the manufacturer's protocol. The resulting cDNA was then diluted, mixed with 2X RT² SYBR-Green PCR Master Mix (SABiosciences), and loaded into the wells of a PCR array plate for PCR amplification and detection. Data analysis was performed using the web-based software package (<http://pcrdataanalysis.sabiosciences.com/mirna/arrayanalysis.php>) for the miRNA PCR array system.

Reverse transcription-quantitative PCR (RT-qPCR). To validate the microarray results, six differentially expressed miRNAs (upregulated: miR-34a, miR-143 and miR-125b; downregulated: miR-32, miR-100 and let-7a) were analyzed using RT-qPCR. Total RNA was isolated from cultured PBLs using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and RNA was treated with RNase-free DNase I, and 3 μg of total RNA was subjected to cDNA synthesis. First-strand cDNA was generated using the PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). The reaction condition was as follows: Pre-denaturation at 95°C for 30 sec, denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec, extension at 70°C for 20 sec, 40 cycles in total. The detection was done using SYBR Green PCR MasterMix (Qiagen, Inc., Valencia, CA, USA) in an Applied Biosystems 7500 instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). U6 was used as the internal reference of miR-34a. Relative quantification was conducted using the $2^{-\Delta\Delta\text{C}_q}$ method (16). Each experiment was repeated for 3 times. The primer sequences used were as follows: miR-34a, forward 5'-CGTCACTCT AGGCTTGGA-3', reverse 5'-CATTGGTGCTTGTCT-3'; and U6, forward 5'-CTCGCTTCGGCAGCACACA-3' and reverse 5'-AACGCTTCACGAATTTGCGT-3'.

Cell proliferation assay. Cell proliferation was evaluated using the Cell Counting Kit (CCK)-8 (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. Briefly, PBLs were seeded in 96-well plates at a density of 1×10^4 cells/well for 24 h at 37°C . miR-34a inhibitor and miR-NC were pretreated for 1 h and TMZ was then added to overnight-cultured cells for another 24, 36 and 48 h. Subsequently, 10 μl CCK-8 solution was added to the culture medium, and incubated for 2 h. Absorbance was measured at a wavelength of 450 nm with a reference wavelength of 570 nm.

Apoptosis assay. PBLs were seeded in 6-well plates at a density of 1×10^5 cells/well for 24 h at 37°C . miR-34a inhibitor or miR-NC were pretreated for 1 h and TMZ was then added to overnight-cultured cells for another 24 h. Subsequently, apoptosis was detected by flow cytometry (FCM) using an Annexin V/fluorescein isothiocyanate (FITC) and propidium iodide (PI) apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) following transfection. Briefly, adherent cells were harvested and suspended in Annexin-binding buffer (1×10^6 cells/ml). Subsequently, cells were incubated with Annexin V-FITC and PI for 15 min at room temperature in the dark and were immediately analyzed by a FACS Aria flow cytometry (BD Biosciences, San Jose, CA, USA).

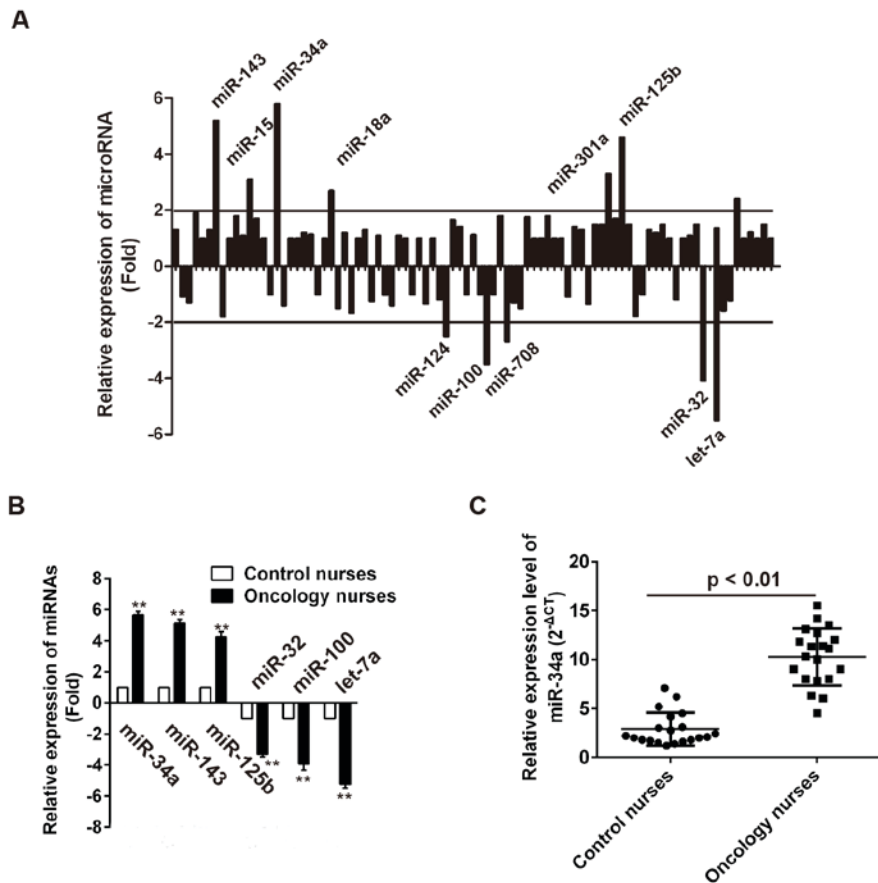


Figure 1. Differential expression of miRNAs between oncology nurses and control nurses. (A) Differential expression of miRNAs between oncology nurses and control nurses, as determined using a microRNA PCR array. (B) Differential expression of 6 miRNAs was verified in 3 paired samples using RT-qPCR. ** $P < 0.01$ vs. control nurses. (C) RT-qPCR analysis of miR-34a expression in 20 paired samples. $P < 0.01$ compared with the normal group, as determined by Student's t-test. Data are presented as the mean \pm standard deviation. miR/miRNA, microRNA; PCR, polymerase chain reaction; RT-qPCR, reverse transcription-quantitative PCR.

Western blot analysis. Total proteins were extracted from PBLs following transfection of miR-34a inhibitor combined with TMZ treatment using radioimmunoprecipitation assay lysis buffer (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and protein concentration was quantified using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). Whole protein extracts were separated by 10% SDS-PAGE and were electrotransferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA), which were then blocked in 5% non-fat dry milk in Tris-buffered saline containing Tween (pH 7.5; 100 mM NaCl, 50 mM Tris and 0.1% Tween-20). The membranes were immunoblotted with specific primary antibodies against caspase-3 (cat. no. sc-7272; 1:1,000), caspase-9 (cat. no. sc-56073; 1:1,000), B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax) (cat. no. sc-70407; 1:1,500) and Bcl-2 (cat. no. sc-56015; 1:1,000; all Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C overnight, followed by horseradish peroxidase-labeled secondary antibody (cat. no. sc-2355; 1:1,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The blots were visualized by chemiluminescence (EMD Millipore, Billerica, MA, USA). Anti- β -actin (Rabbit polyclonal; ab8227; 1:1,000; Abcam, Cambridge, MA, USA) was used as a protein-loading control. Protein band intensity was semi-quantified using Quantity One Basic

software version 4.5.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Statistical analyses were performed with SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean \pm standard deviation of 3 independent experiments. One-way analysis of variance or two-tailed Student's t-test was used for comparisons between groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miRNA expression profile in PBLs from oncology and control nurses. The miRNA PCR array exhibited systematic variations in miRNA expression in PBLs from oncology nurses (Fig. 1A). To validate the microarray analysis findings, 6 miRNAs were selected from the identified differential miRNAs and their expression was analyzed using RT-qPCR in the 20 paired samples. The results confirmed that miR-34a, miR-143 and miR-125b were overexpressed in samples from oncology nurses, whereas the expression levels of miR-32, miR-100 and let-7a were decreased (Fig. 1B). Therefore, these findings indicated that a set of miRNAs is frequently aberrantly expressed in PBLs from oncology nurses. Notably, the expression of

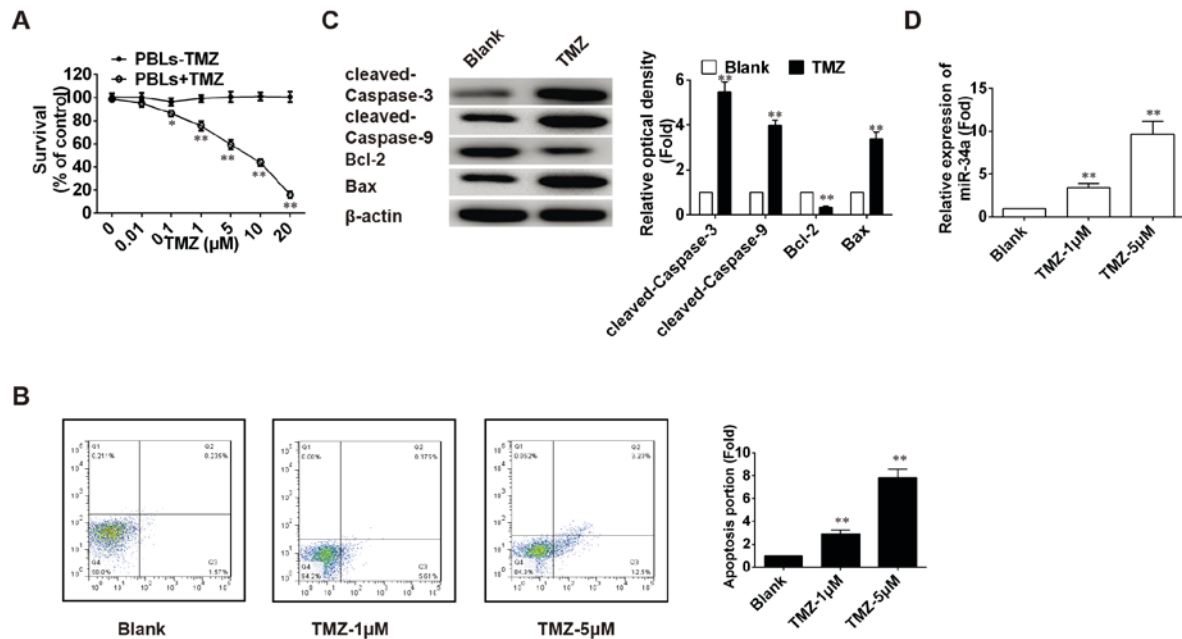


Figure 2. TMZ inhibits cell proliferation, promotes cell apoptosis and positively regulates the expression of miR-34a. (A) Effects of TMZ on proliferation of PBLs. Values are provided as a percentage of untreated control cells (Blank group). Data are presented as the mean + standard deviation. * $P < 0.05$, ** $P < 0.01$ vs. Blank group. (B) Flow cytometry was used to examine cell apoptosis. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. Blank group. (C) Expression of apoptosis-associated proteins, caspase-3, caspase-9, Bax and Bcl-2, was analyzed by western blotting. β -actin was used as an internal control. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. Blank group. (D) miR-34a expression was detected by reverse transcription-quantitative polymerase chain reaction in PBLs following treatment with various doses of TMZ. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. Blank group. Bax, B-cell lymphoma 2-associated X protein; Bcl-2, B-cell lymphoma 2; miR, microRNA; PBLs, peripheral blood lymphocytes; TMZ, temozolomide.

miR-34a exhibited the greatest alteration between oncology nurses and control nurses ($P < 0.01$; Fig. 1C). These data indicated that miR-34a may be abnormally expressed in nurses occupationally exposed to antineoplastic drugs.

Effects of TMZ on PBL proliferation and apoptosis in vitro. To investigate whether TMZ suppresses the growth of normal cells in the body, PBLs were incubated with various doses of TMZ, and proliferation was assessed using the CCK-8 assay. TMZ was able to inhibit the growth of PBLs in a dose-dependent manner (Fig. 2A). Following treatment with 20 μ M TMZ, PBL growth was markedly inhibited.

To determine whether apoptosis contributed to cell growth inhibition, PBLs were incubated with various doses of TMZ, and apoptosis was assessed. As presented in Fig. 2B, TMZ was able to promote apoptosis. Furthermore, the expression levels of proteins involved in apoptotic regulation were detected. TMZ decreased the expression of the anti-apoptotic protein Bcl-2, and increased the expression of the proapoptotic proteins caspase-9, caspase-3 and Bax (Fig. 2C).

In addition, the present study examined the association between miR-34a and TMZ. As presented in Fig. 2D, the expression levels of miR-34a were upregulated in PBLs treated with various doses of TMZ. These data indicated that miR-34a expression may be positively regulated by TMZ.

miR-34a contributes to PBL proliferation and apoptosis in vitro. To evaluate the effects of miR-34a on cellular functions, negative control or miR-34a mimics were transfected into PBLs. Cells transfected with miR-34a mimics exhibited increased miR-34a expression compared with

negative control mimic-transfected cells (Fig. 3A). In addition, miR-34a mimics inhibited cell proliferation in a time-dependent manner, as determined using the CCK-8 assay (Fig. 3B). These results suggested that miR-34a may serve a physiological role in regulating cell proliferation. Subsequently, the present study examined whether cell apoptosis was affected by the overexpression of miR-34a, as determined by FCM. Overexpression of miR-34a significantly increased the rate of apoptosis (Fig. 3C). To determine how miR-34a serves a role in apoptosis, the protein expression levels of caspase-3, caspase-9, Bax and Bcl-2 were analyzed by western blotting. As presented in Fig. 3D, the protein expression levels of caspase-3, caspase-9 and Bax were significantly increased, whereas Bcl-2 was significantly decreased following transfection with the miR-34a mimics. These results demonstrated that miR-34a may inhibit PBL proliferation and induce apoptosis.

Effects of miR-34a knockdown on the cytotoxic activity of TMZ in PBLs. In order to explore the role of miR-34a in PBLs, PBLs transfected with miR-34a inhibitor and NC inhibitor were used as control groups, respectively. Following transfection, RT-qPCR was conducted to examine the miR-34a expression levels. As demonstrated in Fig. 4A, transfection with miR-34a inhibitor significantly decreased the miR-34a expression level compared with the NC inhibitor group. To evaluate the effects of miR-34a on the cytotoxic activity of TMZ (5 μ M) in PBLs, miR-34a inhibitor and inhibitor NC respectively were transfected into PBLs 1 h prior to TMZ treatment. The CCK-8 assay was performed to examine cell proliferation. The results of CCK-8 showed that TMZ significantly reduced the PBLs

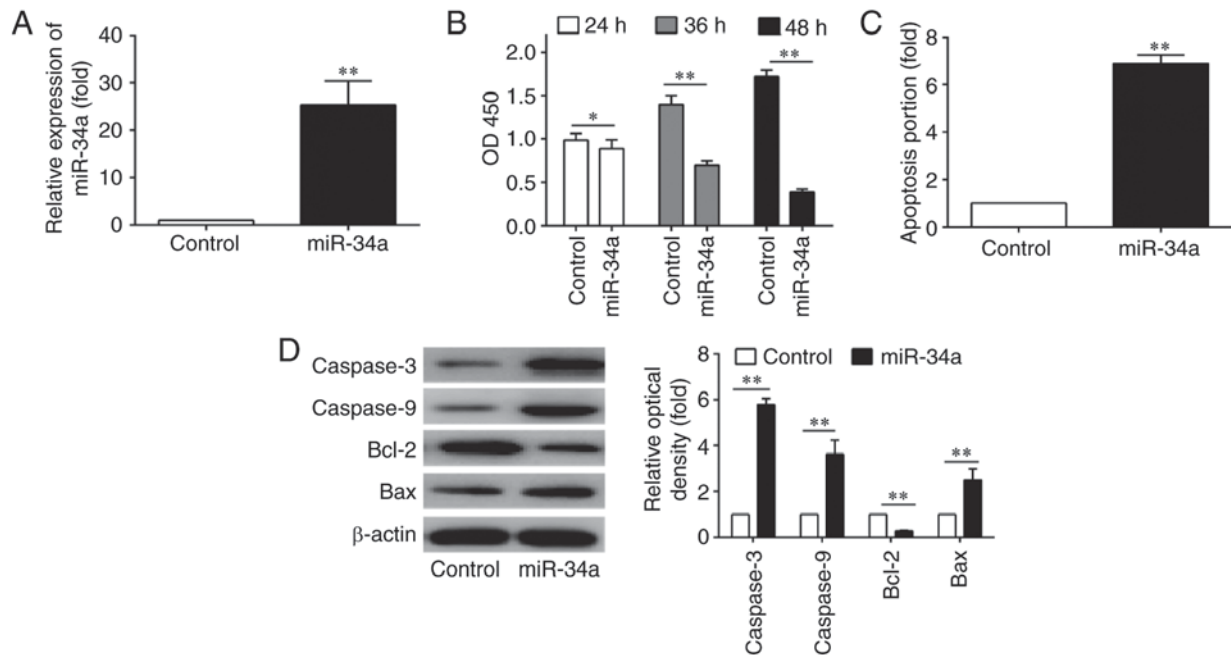


Figure 3. Overexpression of miR-34a induces PBL apoptosis *in vitro*. (A) Efficiency of miR-34a overexpression was determined in PBLs transfected with miR-34a mimics by reverse transcription-quantitative polymerase chain reaction. Control cells negative control mimic-transfected cells. (B) Proliferation of PBLs transfected with miR-34a mimics was measured using the Cell Counting Kit-8 kit. (C) Flow cytometry was used to analyze apoptosis of miR-34a mimics-transfected cells stained with Annexin V-fluorescein isothiocyanate and propidium iodide. (D) Expression levels of apoptosis-associated proteins in miR-34a mimics-transfected cells, caspase-3, caspase-9, Bax and Bcl-2, were determined by western blotting. β -actin was used as an internal control. Data are presented as the mean \pm standard deviation of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. the control group. Bax, B-cell lymphoma 2-associated X protein; Bcl-2, B-cell lymphoma 2; miR, microRNA; OD, optical density; PBLs, peripheral blood lymphocytes.

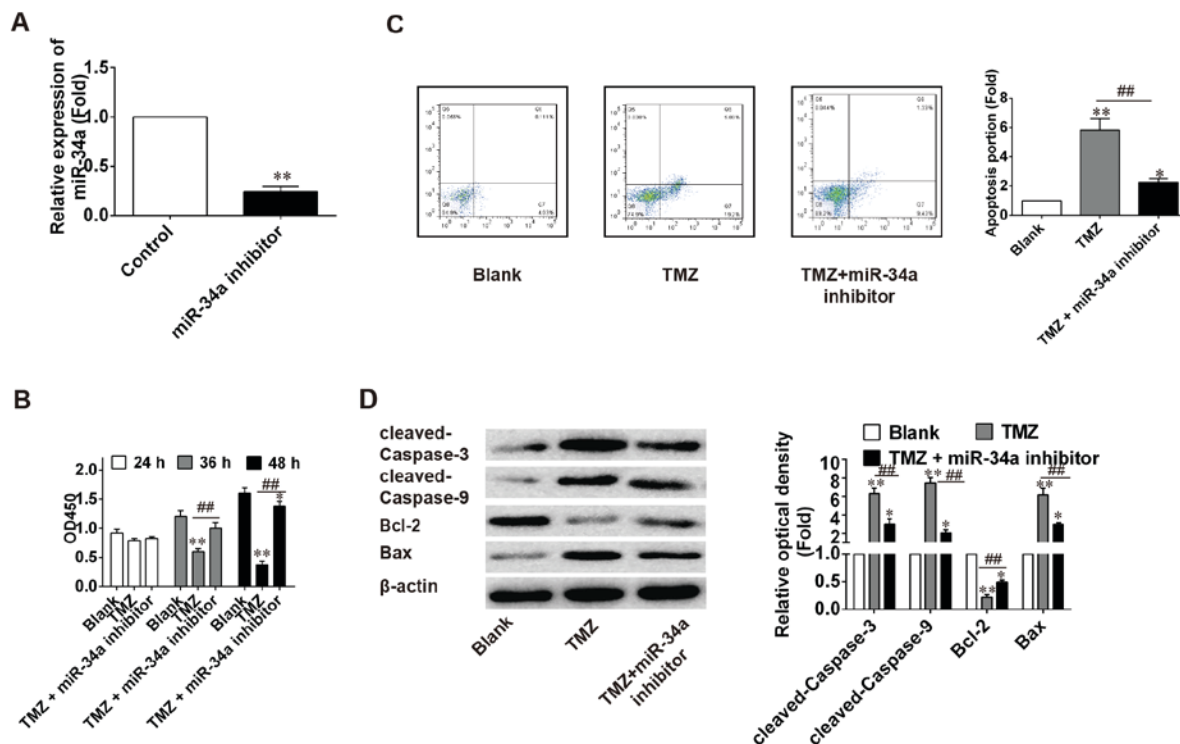


Figure 4. Effects of miR-34a knockdown on the cytotoxic activity of TMZ in PBLs. (A) Efficiency of miR-34a silencing was determined in PBLs transfected with a miR-34a inhibitor by reverse transcription-quantitative polymerase chain reaction. ** $P < 0.01$ vs. the control group. (B) Cell Counting kit-8 assay was performed to detect proliferation of PBLs. miR-34a silencing markedly promoted the proliferation of PBLs pretreated with TMZ. (C) Apoptosis was assessed using flow cytometry. Representative quadrant figures are presented. Knockdown of miR-34a decreased the number of total apoptotic cells in PBL cells pretreated with TMZ. (D) Expression levels of apoptosis-associated proteins, caspase-3, caspase-9, Bax and Bcl-2, were analyzed by western blotting. Untreated cells used as blank group. β -actin was used as an internal control. * $P < 0.05$, ** $P < 0.01$ vs. the blank group, ## $P < 0.01$ vs. the TMZ group. Data are presented as the mean \pm standard deviation from three independent experiments. Bax, B-cell lymphoma 2-associated X protein; Bcl-2, B-cell lymphoma 2; miR, microRNA; OD, optical density; PBLs, peripheral blood lymphocytes.

proliferation, whereas knockdown of miR-34a reversed the inhibitory effect of TMZ on PBLs proliferation (Fig. 4B). This finding suggested that knockdown of miR-34a suppressed the cytotoxic activity of TMZ in PBLs.

To assess the effects of miR-34a knockdown on apoptosis, miR-34a inhibitor-transfected cells were exposed to TMZ, and the effects of TMZ on cell apoptosis were evaluated by FCM. Compared with the TMZ group, knockdown of miR-34a reduced the apoptosis of PBLs following treatment with TMZ (Fig. 4C). Furthermore, knockdown of miR-34a decreased the expression levels of caspase-3, caspase-9 and Bax, and increased the expression of Bcl-2 in PBLs following treatment of TMZ. These results suggested that miR-34a downregulation may reduce cell apoptosis; therefore, miR-34a may enhance the antiproliferative effects of chemotherapeutic agents, including TMZ.

Discussion

The present study identified a small number of miRNAs that are aberrantly expressed in PBLs from nurses occupationally exposed to antineoplastic drugs. Functional assays revealed that miR-34a overexpression decreased cell proliferation and induced apoptosis *in vitro*. Furthermore, silencing miR-34a suppressed the cytotoxic activity of TMZ in PBLs. These findings indicated that altered levels of miR-34a may be considered a novel, minimally invasive biological indicator of occupational exposure of oncology nurses to antineoplastic drugs.

Antitumor drugs have been widely used in clinical tumor therapy as the incidence rate of tumors has increased. However, the majority of antineoplastic agents exhibit genotoxic, mutagenic or carcinogenic effects, and may kill or suppress the growth of normal cells in the body whilst exerting their effects on tumor cells. For example, some antineoplastic drugs, including alkylating agents, antimetabolites, antibiotics and hormones, which are used to treat various types of cancer have been classified as carcinogenic to humans due to their mutagenic and clastogenic properties (17). Although safety standards for cytotoxic drug handling have considerably improved over the past two decades, contamination via inhalation cannot be completely excluded among nurses (18-20).

Previous studies have suggested that miRNAs from blood samples can be used as biomarkers for the identification of disease, injury and drug-induced toxicity (21,22). In addition, several miRNAs have been reported to possess predictive value for the TMZ response in glioblastoma (23-29). The present study investigated whether characteristic miRNA signatures in PBLs could confirm environmental toxin exposure. PBLs were chosen as they can be easily obtained via a minimally invasive route, and they are the cells most often used in human biomonitoring studies. Furthermore, lymphocytes have a half-life of 3-6 months and circulate throughout the body, integrating genotoxic events across body tissues. Forrest and McHale previously compared global gene expression in the peripheral blood mononuclear cells from 6-8 pairs of unexposed controls and workers exposed to high levels of benzene, and identified potential biomarkers of exposure and mechanisms of toxicity (30,31).

The present study focused on miR-34a, which exhibited the greatest alteration between oncology nurses and control nurses, and the results demonstrated that in addition to inhibiting PBL growth and inducing apoptosis, TMZ was also able to positively regulate the expression of miR-34a. The effects of TMZ-altered miRNAs in PBLs from nurses occupationally exposed to TMZ require further investigation.

Functional assays indicated that miR-34a significantly reduced the growth of PBLs and induced a dose-dependent increase in apoptosis. The underlying mechanisms were revealed to include downregulation of the anti-apoptotic protein Bcl-2, and activation of caspase 3/9, thus suggesting that miR-34a may trigger apoptosis via activation of the mitochondria-associated apoptotic pathway. Furthermore, silencing miR-34a suppressed the cytotoxic activity of TMZ in PBLs.

In conclusion, the results of the present study demonstrated that characteristic molecular signatures associated with TMZ exposure exist in PBLs, and these molecular signatures may be used as a predictable and discernible surrogate marker for determining the miRNAome response to TMZ exposure in the environment. Although a small sample size was tested in the present study, these observations warrant further study to validate this alternative testing strategy, which may help to reduce the risk for contamination among exposed workers. In addition, further studies regarding these miRNA signatures are required to provide mechanistic insight into the toxicological effects of TMZ on circulation.

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