Overexpression of MiR-138 Inhibits Cell Growth and Induces Caspase-mediated Apoptosis in Acute Promyelocytic Leukemia Cell Line

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Dysregulated expression of miRNAs can play a vital role in pathogenesis of leukemia. The shortened telomere length, and elevated telomerase activity in acute promyelocytic leukemia cells are mainly indicative of extensive proliferative activity. This study aimed to investigate the effect of overexpression of miR-138 on telomerase activity, and cell proliferation of acute promyelocytic leukemia NB4 cells. MiR-138 was overexpressed in NB4 cells using GFP hsa-miR-138-expressing lentiviruses. hTERT mRNA and protein expression levels were assessed by qRT-PCR and western blot analysis. For evaluation of apoptosis, annexin-V staining and activation of caspases were assessed using flow cytometry and western blot analysis, respectively. Our data demonstrate that overexpression of miR-138 attenuated the hTERT mRNA and protein expression levels. In addition, cell growth was inhibited, and malignant cells underwent caspase mediated-apoptosis in response to miR-138 overexpression. These findings suggest that loss of miR-138 expression may be associated with increased telomerase activity in NB4 cells. Therefore, strategies for up-regulation of miR-138 may result in inhibition of malignant cell growth, and provide a promising therapeutic approach for acute promyelocytic leukemia.

Key words: Apoptosis, caspase, hTERT, miR-138, poly ADP ribose polymerase (PARP)

Acute promyelocytic leukemia (APL), is a distinct subtype of acute myeloid leukemia (AML) characterized by accumulation of promyelocytes in the bone marrow, and peripheral blood (1). The disease is considered to be the most curable subtype of AML, and occurs most often in adults at about 40 years of age (2). Treatment with all-transretinoic acid (ATRA) and As2O3 induces complete remission in APL patients (3). However, relapse/refractory patients showing resistance to ATRA and/or As2O3 are recognized as a clinically significant problem (4). Thus, identification of biological molecules implicated in the proliferation, and survival of acute promyelocytic leukemia cells

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MiRNAs are used as important components of a fat. Telomerase activity is suppressed by miR-138 expression on viruses, were Apoptosis Induction in NB4 Cells by MiR-138 Overexpression.

In the present study, we sought to investigate the therapeutic potential of miR-138 in APL-derived NB4 cells. To achieve this goal, we overexpressed miR-138 in NB4 cells using lentiviral vectors. Our findings revealed that overexpression of miR-138 inhibits cell growth and induces apoptosis in NB4 cells.

Materials and methods

Cell culture

NB4 cells (obtained from Pasteur Institute, Tehran, Iran) were cultured in RPMI-1640 and 10% fetal bovine serum (FBS) with penicillin/streptomycin in an incubator with 5% CO2 and 95% humidity (Memert, Germany). The HEK 293T cells (provided kindly by Dr Frank Grosveld, Erasmus MC) used for the production of lentiviruses, were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS, and penicillin-streptomycin. NB4 cells were divided into 3 groups including non-transduced cells (untreated), cells transduced with GFP-expressing lentiviruses as negative control (blank), and cells transduced with GFP hsa-miR-138-expressing lentiviruses.

MTT assay

The effect of miR-138 expression on cell proliferation was assessed by MTT colorimetric method. Briefly, miR-138-transduced NB4 cells, negative control (blank), and non-transduced NB4 cells were seeded into a 96-well culture plate at a density of 10×10^3 cells/well, and incubated for 0, 24, 48, 96, and 120 h. After removing the medium, cells were incubated with MTT solution (5 mg/ml in PBS) for 4 h, and the resulting formazan was solubilized with DMSO (100 µl). The absorbance of each well was measured at 570 nm in an ELISA reader (BioTek, Vermont, USA) (25).

Apoptosis assay

To investigate the effect of overexpression of miR-138 on NB4 cell death, apoptosis assay was done using Annexin-V Apoptosis Detection Kit II (Roche, Germany) according to the manufacturer’s
instructions. In brief, 0.4 × 10⁶ cells (untreated cells, cells transduced with GFP expressing lentiviruses, and cells transduced with miR-138 expressing lentiviruses) were collected, and washed with PBS, and stained with phycoerythrin (PE). Then, percentage of apoptotic cells was quantified using Becton–Dickinson FACS. Annexin V-positive cells were considered to be in early apoptotic phase.

**Telomerase activity assay**

To investigate the effect of overexpression of miR-138 on telomerase activity, we used the TeloTAGGG Telomerase PCR ELISA kit (Roche, Germany), according to the manufacturer’s instructions. The kit method is a photometric enzyme immunoassay for the detection of telomerase activity, utilizing the telomeric repeat amplification protocol (TRAP). In brief, cells were seeded into 6-well cell culture plates at a density of 1x10⁶ cells/well. Then miR-138-transduced cells along with untreated and blank cells were lysed in lysis buffer and protein extracts were subjected to TRAP assay. The kit includes specific telomere primers bound to biotin, which allows measuring the PCR-amplified telomerase products (and the telomerase activity) by ELISA (26).

**Lentivirus production**

The night before transfection, 3 × 10⁶ HEK-293T cells were seeded into 10 cm dishes. After one day, HEK293T cells were transfected with GFP hsa-miR-138-expressing lentiviral vectors, and GFP-expressing lentiviral vectors as a control vector along with packaging vectors (psPAX2 and pMD2.G) using FuGENE-6 transfection reagent (Promega, USA). Lentiviral supernatants were harvested at 48 and 72 h after transfection, and filtered through 0.45 μm PVDF filters. The supernatant were then concentrated by ultracentrifugation (2 h at 100,000 g) in Beckman Optima L-90K ultracentrifuge (Beckman Coulter, USA). The virus-containing pellet was dissolved in DMEM, aliquoted, and stored at -80 °C.

**Infection of target cells with lentivirus**

NB4 cells were infected by adding 1 ml of concentrated virus supplemented with 2 μg/ml polybrene to 5 × 10⁶ cells in 24-well plates. The viral supernatant was replaced with standard growth medium after 36 h, and transduction efficiency was monitored by GFP expression at 96 h after replacement of the virus-containing medium with normal growth medium.

**RNA extraction and qRT-PCR**

Total RNA was extracted from the cells transduced with miR-138-expressing lentiviruses, cells transduced with GFP-expressing lentiviruses (blank), and untreated cells using TriPure isolation reagent (Roche, Germany), according to the manufacturer’s instruction. One microgram of isolated RNA was used for the preparation of cDNA using Revert Aid First Strand cDNA Synthesis kit (Thermo Scientific, Waltham, Massachusetts, USA). The prepared cDNA was subjected to quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR), using Maxima SYBR Green Master mix (Thermo Scientific, Waltham, Massachussets, USA) in the Rotor Gene 6000 Real Time PCR instrument (Corbett Research, Hilden, Germany). DNA was amplified in a 40-cycle PCR reaction with the following conditions: denaturation at 95 °C for 15 s, annealing and elongation at 60 °C for 60 s. The fold induction or repression was measured relative to control, and calculated after adjusting for reference gene GAPDH (25). Each sample was analyzed in triplicate. qRT-PCR was performed with hTERT specific primers (forward primer: 5'- atgcgacagttcgtggctca-3' and reverse primer: 5'-gtcggagtc-3') and reverse primer: 5'-gaaggtgaagatcctggacagtga-3'). GAPDH was amplified using the following primers: forward: 5'-gaaggtgaagatcctggacagtga-3' and reverse: 5'-gaaggtgaagatcctggacagtga-3'.

**Western blot analysis**

Cells that were transduced with GFP hsa-miR-138-expressing lentiviruses, untreated cells, and cells transduced with GFP-expressing lentiviruses were centrifuged, and cellular pellets were washed.
with cold PBS and lysed (5 × 10⁶ cells/ aliquots) in 0.2 ml of RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 0.5% sodium deoxycholate) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich, Missouri, USA). After centrifugation at 13,000 g for 20 min at 4 °C, the supernatant was collected. Protein concentrations were determined by Bradford protein assay, and equivalent amounts of total cellular protein were separated by 10% SDS-PAGE, according to the method of Laemmli. The gels were then electroblotted onto nitrocellulose membranes (Hybond-ECL, Amersham Corp). Subsequently, membranes were blocked with 5% nonfat dry milk in TBS containing 0.1% (v/v) Tween-20 for 1 h at room temperature, and probed with specific primary antibodies overnight at 4 °C. Primary antibodies including caspase-3, caspase-9, cleaved PARP, cyclin D3, hTERT and β-actin were obtained from Cell Signaling Technology (Danvers, Massachusetts, USA). After 3 washes in TBS-T, membranes were incubated with HRP-conjugated secondary antibodies (Santa Cruz, California, USA). Proteins were then visualized with a chemiluminescence detection system (Amersham ECL Advance Kit, GE Healthcare) (25).

Statistical analysis

Data were analyzed using two-tailed student t-test. A P value <0.05 was considered to be significant.

Results

Overexpression of miR-138 inhibits cell growth in NB4 cells

NB4 cells were transduced with either GFP hsa-miR-138-expressing lentiviruses or GFP-expressing lentiviruses as a blank or negative control, and then transduction efficacy was evaluated with fluorescent microscopy (Figure 1A). The effect of miR-138 overexpression on cell metabolic activity was investigated using MTT assay at 0, 24, 48, 72, 96 and 120 h after removal of the lentivirus-containing medium. As shown in Figure 1B, transduction of cells with miR-138 reduced cell viability in NB4 cells.

hTERT expression and telomerase activity are inversely correlated with miR-138 overexpression in NB4 cells

We focused on the hTERT, a potential target gene of miR-138 (27). It has been reported that hTERT mRNA is overexpressed in AML patients (28), and it has been shown that hTERT is necessary to prevent apoptosis, and induce cell proliferation (15, 29). We measured hTERT mRNA

Figure 1A

Fig. 1. Overexpression of miR-138 suppresses the growth of APL derived cell line, NB4. A: transduction efficacy was assessed by evaluation of GFP expression at 72 h after removal of the lentivirus-containing medium; B: cell viability was measured using MTT assay at 0, 24, 48, 72, 96 and 120 h after removal of the virus-containing medium (n=3; *P<0.05 compared to non-transduced NB4 cells).
and protein expression in NB4 cells transduced with GFP hsa-miR-138- and GFP-expressing lentiviruses (blank). In addition, we assessed hTERT mRNA and protein in non-transduced NB4 cells. To this end, at 96 h after removal of the virus containing medium, cells were subjected to qRT-PCR and western blot analysis. As seen in Figure 2A and Figure 2B, hTERT mRNA and protein expression level were significantly reduced in cells transduced with miR-138-expressing lentiviruses compared with untreated and blank cells. Additionally, mRNA and protein expression of hTERT were compared with each other and an inverse correlation between miR-138 overexpression and hTERT mRNA and protein expression was found (Figure 2C). Next, we performed TRAP assay to investigate the effect of miR-138 overexpression on telomerase activity. Figure 2D demonstrates that, telomerase activity for NB4 cells reduced approximately 35% upon overexpression of miR-138.

**Overexpression of miR-138 triggers caspase-mediated apoptosis in NB4 cells**

To investigate the effects of miR-138 overexpression on apoptosis, NB4 cells were transduced with lentiviruses expressing miR-138, and modulation of phosphatidylserine externalization was evaluated by Annexin-V binding assay. As seen in Figure 3A, overexpression of miR-138 resulted in increased percentage of Annexin-V positive cells. Moreover, to ascertain if caspase cascade activation can play a role in miR-138 overexpression-induced apoptosis, we investigated the cleavage of PARP or activation of caspase-9 and caspase-3 by western blotting at 96 h after removal of the lentivirus-containing medium. Moreover, we assessed cyclin D3 protein expression through western blotting. As presented...
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in Figure 3B, expression of cyclin D3 was reduced after overexpression of miR-138 and cleaved products of caspases-9, caspase-3 and PARP were seen in cells transduced with GFP hsa-miR-138-expressing lentiviruses compared with cells transduced with GFP-expressing lentiviruses and non-transduced NB4 cells. These findings suggest that overexpression of miR-138 induces caspase-mediated apoptosis in NB4 cells.

Discussion

Aberrant miRNA expression plays a crucial role in the pathogenesis of various human malignancies (30). A growing body of evidence indicate that miR-138 functions as a tumor suppressor gene and alteration in its expression is associated with the development and progression of different types of cancers (14, 15). It has been reported that miR-138 is downregulated in primary CML samples and K562 cells which can be restored by imatinib treatment (31). Additionally, Ma et al. claimed in their study that overexpression of miR-138 attenuates the proliferation and survival of gallbladder carcinoma cells, and inhibits the growth of tumors in the gallbladder carcinoma xenograft model in nude mice (17). In the present study, we found that overexpression of miR-138 significantly inhibits cell growth and induces apoptosis in acute promyelocytic leukemia NB4 cells. Our data are consistent with a report by Zhao et al. indicating that miR-138 is a master regulator of cell proliferation and apoptosis pathways in leukemia cells (16). Occurrence of apoptosis is associated with activation of multiple caspases such as caspases-9 and -3 that are the key executors of apoptosis (32). Previous study by Ma et al. demonstrated an increased cleavage of caspase-3 in the miR-138-overexpressing gallbladder carcinoma cells (17). Likewise, Zhu et al. reported that miR-138 acts as a tumor suppressor which can enhance cisplatin-induced apoptosis via caspase-3 activation in osteosarcoma cells (13). In line with previous studies, our data showed that overexpression of miR-138 induces apoptosis in NB4 cells by

Fig. 1. Cell death was induced through caspase-mediated apoptosis upon upregulation of miR-138 in NB4 cells. A: to evaluate the effect of miR-138 on apoptosis, 96 h after removal of the virus-containing medium, transduced and non-transduced cells were analyzed for Annexin-V by flowcytometry; B: whole cell lysates from cells transduced with GFP hsa-miR-138-expressing lentiviruses, and non-transduced NB4 cells were prepared at 96 h after removal of the lentivirus-containing medium. Protein expression levels of caspase 3, 9, PARP and Cyclin D3 were analyzed by western blot. Equal sample loading was verified by β-actin.
activation of caspase-9, -3 and cleavage of PARP protein. It is well known that cyclin D3 is necessary for cell cycle progression in G1 phase (32). Over-expression of cyclin D3 has been implicated in the pathogenesis of various types of cancers (33, 34). Importantly, Kato et al. showed that overexpression of cyclin D3 induces proliferation and blocks differentiation of myeloid precursor cells (35).

It has been reported that cyclin D3 is a direct target of miR-138, and upregulation of this microRNA can induce cell cycle arrest in hepatocellular carcinoma cells (14). Moreover, Xu et al. showed that cyclin D3 would be repressed upon miR-138 overexpression which binds to 3'-UTR region of cyclin D3 (31). In agreement with these studies, we found downregulation of D3 protein level in NB4 cells upon overexpression of miR-138 (Figure 3B). Previous studies demonstrated that upregulation of hTERT can play a critical role in carcinogenesis, and immortalize cells through telomerase-dependent manner (36, 37). Non-coding RNAs have been implicated in the post-transcriptional regulation of hTERT expression. Interestingly, Mitomo et al. reported that hTERT is a potential target gene of miR-138 (20). They demonstrated that miR-138 can directly bind its target site in the hTERT 3'UTR, and repress hTERT protein expression in human anaplastic thyroid carcinoma cell lines. Further, recent study by Chao et al. has shown that hTERT mRNA and protein expression levels were reduced upon overexpression of miR-138 in malignant myeloma cells (15). Consistent with previous findings, we also indicated that overexpression of miR-138 is correlated with downregulation of hTERT mRNA and protein levels in NB4 cells. Moreover, telomeric repeat amplification protocol (TRAP) assay revealed significant reduction in telomerase activity of miR-138-overexpressing APL cells.

In conclusion, our findings indicate that overexpression of miR-138 is able to inhibit cell growth, and induce apoptosis in NB4 leukemia cells through down-regulation of cell cycle regulatory molecule cyclin D3 and suppression of telomerase activity. Therefore, restoration of miR-138 expression can be explored as a potential therapeutic strategy for APL treatment.

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Conflict of interest
The authors declare no conflict of interest.

References
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