Research Article

miR-146a Expression Level as a Novel Putative Prognostic Marker for Acute Promyelocytic Leukemia

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Background. Although the curative rate for acute promyelocytic leukemia (APL) has been improved over decades, long-term prognosis is still poor. The genetic pathways that regulated cell lineage fate during the development of APL remain unclear. Methods. We investigated the correlations of miR-146a expression with its target gene Smad4 and the biological behaviors of NB4 cells. We also analyzed their expression in clinical samples from APL patients. Results. miR-146a influenced apoptosis and proliferation in NB4 cells. miR-146a influenced endogenous Smad4 protein levels in APL cells. miR-146a expression levels were positively correlated with white cell counts and PML/RARα fusion protein expression. miR-146a expression levels were negatively correlated with Smad4 protein and the helper T cell (Th)/the suppressor T cell (Ts) ratio in these patients. Conclusions. These findings indicated that miR-146a played an important role in the development of APL in part through the repression on Smad4 protein expression. miR-146a functioned as an oncogene and may be a novel prognostic biomarker in APL.

1. Introduction

Acute promyelocytic leukemia (APL) has been identified as an M3 subtype of acute myelogenous leukemia (AML) by the French-American-British (FAB) classification. APL is characterized by maturation arrest at the promyelocytic stage [1]. A specific t (15;17) chromosomal translocation encodes a promyelocytic leukemia (PML) and retinoic acid receptor-α (RARα) fusion protein to form PML/RARα, an oncogenic protein found in approximately 10%–25% of adults with AML [2]. PML/RARα interferes with the process of myeloid differentiation by repressing the transcription of retinoid acid- (RA-) responsive genes.

Although the outcomes of APL have been dramatically improved since the successful introduction of all-trans retinoic acid (ATRA), arsenic acid, and combined anthracycline-based chemotherapy, more than 10% of newly diagnosed APL patients die of the disease; moreover, the 5-year cumulative incidence of relapse is around 15% in high-risk subgroups [3]. Furthermore, the specific genes and pathways that regulate lineage fate during APL development remain unclear.

MicroRNAs (miRNAs) are a group of highly conserved non-protein-coding RNAs comprised of about 19–25 nucleotides. miRNAs can regulate the expression of a variety of genes by binding the 3′ untranslated regions (3′ UTRs) of messenger RNAs (mRNAs) in a sequence-specific manner to regulate mRNA translation or degradation in eukaryotic cells [4], ultimately affecting cell proliferation, apoptosis, development, and differentiation [5].

miR-146a was first identified as having a role in the innate immune and inflammatory response to microbial infection [6]. Later studies showed that miR-146a is expressed at relatively high levels in bone marrow (BM) CD34+ progenitors from healthy donors but is found at low levels in AML patients and even lower levels in monocytes, granulocytes, erythrocytes, and megakaryocytes from the peripheral blood or BM of healthy donors [7]. miR-146a is strongly upregulated during megakaryopoiesis in mice [8], and dysregulation of miR-146a has been found in APL cells following retinoic
acid (RA) induction [9–12]. Our previous study confirmed the reduced expression of miR-146a in NB4 cells following treatment with ATRA [13]. However, the role of miR-146a in the clinical progression of APL remains unknown.

In this study, we investigated the correlations of miR-146a expression with the expression of its target gene Smad4 and the biological behaviors of NB4 cells, such as proliferation and apoptosis. To further elucidate the role of miR-146a in APL, we analyzed its expression in samples from 32 APL patients for whom clinical data were available. Interestingly, the expression levels of miR-146a were positively correlated with white blood cell (WBC) counts in the peripheral blood and expression of the PML/RARα fusion protein. miR-146a expression levels were also negatively correlated with Smad4 protein and the helper T cell (Th)/the suppressor T cell (Ts) ratio in these patients. These results indicated that miR-146a functioned as an oncogene in APL and may be a potential biomarker for malignancy.

2. Materials and Methods

2.1. Cell Culture and Transient Transfection with miR-146a Mimics. The human promyelocytic cell line NB4 was a gift from Shanghai Institute of Hematology and was cultured in RPMI 1640 medium with 10% fetal calf serum (FCS; Gibco; BRL, UK) at 37°C in a 5% CO2 humidified incubator. On the day of transfection, 5 × 10⁵ cells/mL were plated in 6-well plates with RPMI 1640 supplemented with 10% FBS. Transfections were carried out with 100 nM DMEM-diluted pre-miR miR-146a precursor, pre-miR negative control, anti-pre-miR miR-146a precursor, or anti-pre-miR negative control (Ambion, Carlsbad, CA, USA) using siPORT NeoFX Transfection Agent (Ambion, Carlsbad, CA, USA). Twenty-four hours after transfection, the medium was replaced, and the cells were cultured for another 24 h. To monitor the transfection efficiency of the miRNAs, pre-miR has-miR-1 precursor and anti-miR has-let-7c miRNA inhibitor were transfected into NB4 cells in parallel experiments according to the manufacturer’s instructions.

2.2. Cell Proliferation, Apoptosis, and Cell Cycle Analyses. Live cell proliferation assays were carried out by trypan blue dye exclusion using a Bio-Rad automatic cell counter (Bio-Rad, Berkeley, CA, USA). The number of NB4 cells was calculated in triplicate after 24 or 48 h of culture. Apoptotic cells were detected with an Alexa Fluor 488 annexin V/Dead cell apoptosis kit (Invitrogen, Carlsbad, CA, USA) by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). Early apoptotic cells were defined as Annexin-V-positive/propiodium iodide (PI)-negative cells. The experiments were repeated 3 times.

2.3. Cell Cycle Distribution. For analysis of the cell cycle distribution, NB4 cells were washed 3 times with cold phosphate-buffered saline (PBS), fixed with 70% ethanol, and incubated at −20°C for more than 12 h. Before examination, the cells were washed with cold PBS and stained with 0.5 mL PI staining buffer (200 mg/mL RNase A and 50 μg/mL PI in PBS). The mixture was incubated at 37°C for 30 min in the dark, and cell cycle distribution was analyzed by flow cytometry (BD Biosciences). The experiments were repeated 3 times.

2.4. Western Blot Analysis. A total of 5 × 10⁶ cells of each experimental group were harvested and subsequently were washed twice with phosphate-buffered saline (PBS). Cells were then lysed with lysis buffer (300 mM NaCl, 0.5% NP-40, 1 mM DTT, 200 mM PMSF, protease inhibitor tablet) for 20 minutes. The protein concentration of the cell lysates was quantified using a bicinchoninic acid (BCA) protein Assay Kit (Kangchen, China). 20 μg protein extracted from each experimental sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes and blotted with rabbit polyclonal anti-Smad4 (1:5000, Cell Signaling Technology, Danvers, MA, USA) or rabbit monoclonal anti-GAPDH (1:2000, Cell Signaling Technology) antibodies. Protein bands were visualized with the use of enhanced chemiluminescence reagent (Pierce), according to the manufacturer’s instructions, and band intensities were analyzed using Bandscan 5.0 (Glyko, Hayward, CA, USA).

2.5. Patients, Sample Collection, and Therapeutic Methods. Thirty-two APL patients and ten iron deficiency anemia patients as control who attended Renji Hospital were enrolled in this study. The clinical characteristics of these patients are shown in Table 1. Bone marrow was collected from patients at diagnosis or after therapy. APL patients were treated with ATRA (30 mg/m²) and daunorubicin (DNR; 60 mg/m²)/idarubicin (IDA; 8 mg/m²) daily for 3 days. Arsenic trioxide was used at 10 mg daily until patients achieved complete remission. The details of these patients including treatment protocol can be found in Table 2. Protein extracts from 14 patients were used for western blotting, including 6 pairs of samples taken before and after therapy. Written informed consent for participation in this study was
2.6. Statistical Analyses. To investigate whether miR-146a expression correlated with clinical quantitative variables (e.g., WBC counts), we used Pearson correlation analysis within SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The Mann-Whitney U test was carried out to assess the differential expression of miR-146a for statistical significance. Data from western blotting were analyzed by paired t-tests using SAS version 12.6. All results were presented as the mean ± standard deviation (SD). P values of less than 0.05 were considered statistically significant.

3. Results

3.1. Ectopic Expression of miR-146a Affected Cell Apoptosis and Proliferation but Did Not Affect the Cell Cycle in NB4 Cells Lines. To examine the functional role of miR-146a in APL cells, we transfected miR-146a into NB4 cells and collected the cells 48 h after transfection. The results showed that forced expression of miR-146a significantly inhibited apoptosis and increased cell viability in NB4 cells, while knockdown of miR-146a expression using an anti-miR-146a inhibitor increased apoptosis and reduced cell viability significantly (Figures 1(a) and 1(b)). Overexpression of miR-146a in NB4 cells did not affect the cell cycle distribution (Figure 1(c)). The expression of miR-146a before and after transfection was confirmed by RTq-PCR (data not shown). Thus, miR-146a may function as an oncogene in leukemogenesis.

3.2. Exogenous Expression of miR-146a Affected Endogenous Smad4 Protein Expression in APL Cells Lines. As shown in our previous research, Smad4 protein expression was repressed by more than 30% in miR-146a-transfected 293T cells [13]. We overexpressed miR-146a to confirm the effects of miR-146a on Smad4 protein expression in NB4 cells. The results showed that miR-146a significantly reduced Smad4 protein levels but did not affect smad4 mRNA expression, as compared with the scramble control group. In contrast, transfection with miR-146a inhibitor increased Smad4 protein (Figures 2(a) and 2(b)), indicating that miR-146a regulated the expression of Smad4 protein in NB4 cells. To confirm the relation in primary leukemia cells, the expression levels of miR-146a and Smad4 were examined in 14 APL samples. The results showed that Smad4 expression was inversely correlated with miR-146a expression in APL samples (Figure 2(c)).

As we previously demonstrated in NB4 cells, ATRA could suppress miR-146a expression, which subsequently increased the expression of Smad4 protein [13]. To study the effects of ATRA on miR-146a and Smad4 expression levels in primary cells, 6 pairs of samples from APL patients before and after ATRA treatment were analyzed. The results showed that miR-146a expression decreased, while Smad4 protein levels increased after treatment with ATRA, as compared to matched samples before therapy (Figure 2(d)).

These data demonstrated that the negative correlation between miR-146a and Smad4 protein level may be an indicator of disease progression and treatment outcomes.

3.3. miR-146a Expression Was Associated with the Clinical Characteristics of Patients. Next, we investigated the correlations between miR-146a expression and patient characteristics, including age, WBC count, blast cell percentage in the bone marrow or peripheral blood, PML/RARα expression levels, and Th/Ts ratios. Compared to the controls, we found average miR-146a expression levels were higher in APL patients (Figure 3(a)). We found a positive correlation between miR-146a expression and peripheral WBC counts (Figure 3(b)). However, no correlations were found between miR-146a expression and age or blast percentage in peripheral or bone marrow (data not shown).

To investigate the correlations between miR-146a and PML/RARα and Th/Ts, 32 samples from patients with APL were examined. The results showed that patients with higher miR-146a expression exhibited higher levels of PML/RARα (Figure 3(c)), but lower Th/Ts ratios (Figure 3(d)).

4. Discussion

Recent studies have mainly focused on analyzing the function of miR-146a in hematopoietic cell differentiation; these data have indicated that miR-146a expression is finely tuned during cell differentiation and miR-146a-mediated expression of target genes plays an important role in this process [15]. Moreover, one study demonstrated that overexpression of miR-146a in mouse hematopoietic stem/progenitor cells results in a transient myeloid expansion in vivo [16]. miR-146a and its predicted target gene Smad4 were identified in a previous study using luciferase assays [13]. In our previous study, miR-146a expression was decreased during retinoid acid induction in NB4 cells, accompanied by upregulation of Smad4 protein. These results suggested that miR-146a may play specific roles in APL genesis or ATRA-induced cell differentiation. Elucidating the role of miR-146a will improve our understanding of the malignant progression of APL.

In our present study, transfection with miR-146a mimics increased proliferation, while inhibition of miR-146a expression resulted in decreased cell growth and increased apoptosis in NB4 cells. However, ectopic expression of miR-146a had no effect on cell cycle in NB4 cells. These findings are consistent with several previous studies. Starczynowski et al. investigated the role of miR-146a in hematopoiesis by using retroviral infection and overexpression of miR-146a.
Table 2: Clinical variables for controls and APL patients.

| Patients ID | Gender | Age | WBC (×10^9/L) | PML/RARα : ABL relative ratio | Th/Ts | Smad4/GAPDH | Therapy          |
|------------|--------|-----|---------------|-------------------------------|------|-------------|------------------|
| Control    |        |     |               |                               |      |             |                  |
| C1         | M      | 44  | 6.32          | NA                            | 0.98 | NA          | NA               |
| C2         | F      | 34  | 4.67          | NA                            | 1.03 | NA          | NA               |
| C3         | F      | 59  | 5.96          | NA                            | 0.61 | NA          | NA               |
| C4         | F      | 28  | 11.30         | NA                            | 1.31 | NA          | NA               |
| C5         | M      | 24  | 8.35          | NA                            | 1.87 | NA          | NA               |
| C6         | F      | 63  | 5.90          | NA                            | 1.55 | NA          | NA               |
| C7         | F      | 30  | 7.40          | NA                            | 1.07 | NA          | NA               |
| C8         | M      | 38  | 3.76          | NA                            | 1.12 | NA          | NA               |
| C9         | F      | 68  | 7.75          | NA                            | 2.05 | NA          | NA               |
| C10        | M      | 35  | 4.81          | NA                            | 2.11 | NA          | NA               |
| APL        |        |     |               |                               |      |             |                  |
| A1         | F      | 53  | 0.80          | 174                           | 2.03 | 1.4061      | ATRA + ATO + DNR |
| A2         | F      | 56  | 3.20          | 285                           | 1.69 | 1.2696      | ATRA + ATO + IDA |
| A3         | F      | 67  | 22.40         | 713.5                         | 1.01 | 0.7683      | ATRA + ATO + DNR |
| A4         | M      | 33  | 6.56          | 493.5                         | 1.49 | 0.9575      | ATRA + ATO + IDA |
| A5         | M      | 76  | 57.44         | 829.5                         | 0.57 | 0.3519      | ATRA + DNR       |
| A6         | F      | 33  | 7.50          | 537                           | 1.39 | NA          | ATRA + DNR       |
| A7         | M      | 64  | 1.50          | 169                           | 1.85 | 1.7688      | ATRA + ATO + IDA |
| A8         | F      | 44  | 2.30          | 416.5                         | 1.88 | 1.3570      | ATRA + ATO + IDA |
| A9         | F      | 34  | 3.80          | 434                           | 1.72 | NA          | ATRA + ATO + IDA |
| A10        | F      | 63  | 6.30          | 501.5                         | 0.97 | 1.1272      | ATRA + ATO + IDA |
| A11        | M      | 28  | 64.60         | 1048                          | 0.36 | 0.2464      | ATRA             |
| A12        | F      | 24  | 1.05          | 225.5                         | 1.95 | NA          | ATRA + ATO + IDA |
| A13        | F      | 24  | 1.60          | 143                           | 1.8  | 2.0794      | ATRA + ATO + IDA |
| A14        | M      | 30  | 7.60          | 221                           | 1.61 | 0.8966      | ATRA + ATO + IDA |
| A15        | F      | 38  | 34.10         | 617                           | 0.49 | NA          | ATRA + DNR       |
| A16        | M      | 21  | 2.30          | 378                           | 1.15 | NA          | ATRA + ATO + DNR |
| A17        | F      | 23  | 0.70          | 183                           | 2    | 1.5293      | ATRA + DNR       |
| A18        | M      | 48  | 2.60          | 331                           | 1.27 | NA          | ATRA + DNR       |
| A19        | F      | 24  | 3.90          | 411                           | 1.86 | NA          | ATRA + ATO + IDA |
| A20        | F      | 34  | 9.70          | 612                           | 1.31 | NA          | ATRA + ATO + DNR |
| A21        | F      | 60  | 1.30          | 173                           | 1.87 | 1.9294      | ATRA + ATO + IDA |
| A22        | F      | 50  | 1.60          | 199                           | 1.91 | NA          | ATRA + ATO + IDA |
| A23        | M      | 30  | 1.60          | 189                           | 1.85 | NA          | ATRA + ATO + IDA |
| A24        | F      | 56  | 1.10          | 191                           | 1.99 | NA          | ATRA + ATO + IDA |
| A25        | M      | 51  | 3.20          | 367                           | 1.7  | NA          | ATRA + ATO + IDA |
| A26        | F      | 24  | 2.20          | 336                           | 1.91 | NA          | ATRA + ATO + IDA |
| A27        | M      | 40  | 1.00          | 156                           | 1.97 | NA          | ATRA + ATO + IDA |
| A28        | M      | 15  | 28.30         | 729                           | 0.93 | NA          | ATRA + ATO + IDA |
| A29        | F      | 62  | 0.60          | 201                           | 2.13 | NA          | ATRA + ATO + DNR |
| A30        | M      | 50  | 41.40         | 854                           | 0.64 | NA          | ATRA + ATO + IDA |
| A31        | F      | 44  | 67.20         | 1137                          | 0.3  | 0.3373      | ATRA + ATO + IDA |
| A32        | M      | 52  | 1.50          | 179                           | 1.82 | NA          | ATRA + ATO + IDA |

M: male; F: female; WBC: white blood cell; ATRA: all trans retinoic acid; ATO: arsenic trioxide; DNR: daunorubicin; IDA: idarubicin; NA: not available.

in mouse hematopoietic stem/progenitor cells, followed by bone marrow transplantations. The transplantation of these cells resulted in a transient myeloid expansion [16]. Moreover, miR-146a has been found to play an antiapoptotic role during T cell activation [17].

Some other results were inconsistent with our results. Overexpression miR-146a in hepatic stellate cell (HSC) inhibited cell proliferation by enhancing cell apoptosis [18]. Treatment HL60 cells with demethylating agents increased miR-146a expression, and forced expression of miR-146a decreases cell
Figure 1: Continued.
Figure 1: The functional role of miR-146a. (a) Forced expression of miR-146a by transfection of cells with pre-miR-146a with lipidosome significantly inhibited apoptosis, whereas downregulation of miR-146a by anti-pre-miR-146a inhibitor significantly increased apoptosis in NB_4 cells. (b) Forced expression of miR-146a significantly increased, whereas downregulation of miR-146a significantly decreased cell proliferation in NB_4 cells. (c) The cell cycle was not affected after ectopic expression of miR-146a. Normalized mean values of 3 independent experiments and standard errors (means ± SDs) are shown. *, *P ≤ 0.05; **, *P ≤ 0.001 (paired t test).

miR-146a expression is significantly downregulated during monocytic differentiation in NB_4 cells in response to chemical inducers vitamin D and phorbol 12-myristate 13-acetate (PMA) [33]. Our previous study showed that miR-146a is downregulated during NB_4 differentiation induced by ATRA [13]. In the current study, miR-146a expression...
was decreased, accompanied by increased Smad4 protein expression in APL patients exhibiting complete remission. These data suggested that miR-146a expression was correlated with APL cell differentiation.

In our analysis, miR-146a was found to be positively correlated with WBC counts in patients, which is consistent with our in vitro data. In another study, miR-146a was overexpressed in hematopoietic stem cells, and the modified cells were then transplanted into recipients’ bone marrow. Transplantation of these cells resulted in a transient myeloid expansion [16]. This result also showed that miR-146a indeed affected cell proliferation. PML/RARα complexes can bind to the predicted PML/RARα binding site in the promoter region of miR-146a, and the repressive effects of PML/RARα complexes can be blocked by ATRA, even in primary blast cells [11]. In our study, expression of the PML/RARα fusion gene was decreased after patients achieved complete remission following retinoid acid induction, and miR-146a expression decreased simultaneously, indicating that miR-146a was regulated by PML/RARα in the leukemogenesis of APL.

miR-146a is abundantly expressed in regulatory T cells and T helper type-1 cells as compared with naïve mature T cells and T helper type-2 [34]. miR-146a contributes to the
regulation of the type I interferon signal transduction pathway, which is important for immune surveillance [17, 21]. Our data showed a negative correlation between miR-146a and the Th/Ts ratio in APL patients (Figure 3(c)), suggesting that overexpression of miR-146a may impair immune surveillance in APL patients.

In conclusion, our data demonstrated that miR-146a played an important role in APL cells by increasing cell proliferation and inhibiting cell apoptosis, at least in part via the tumor-suppressor Smad4. miR-146a expression was associated with peripheral WBC counts, Th/Ts ratios, and PML/RARα expression levels in APL patients, suggesting that miR-146a may represent a novel biomarker for APL and could be used to evaluate the effectiveness of chemotherapy for APL.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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