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ORIGINAL ARTICLE

Aberrant Expression of the miR-181b/miR-222 after Hematopoietic Stem Cell Transplantation in Patients with Acute Myeloid Leukemia

Mahdiyar Iravani Saadi[1] · Nargess Arandi[1] · Ramin Yaghobi[2] · Negar Azarpira[2] · Bita Geramizadeh[2] · Mani Ramzi[1]

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Abstract Recently, dysregulated expression of various micro RNAs has been reported in hematologic malignancies, especially AML disease which affects normal hematopoiesis in these patients and thereby contribute to clinical outcome of AML patients, associated with either poor or favorable prognosis. Herein, we evaluated the expression of miR-181b and miR-222 in acute myeloid leukemia patients and correlation with response to therapy after hematopoietic stem cell transplantation. Eighty newly diagnosed AML patients and 80 healthy controls were recruited. The expression of miR-181b and miR-222 was evaluated by real-time SYBR Green PCR method. miR-181b gene expression was significantly increased (4.7 fold) whereas miR-222 was decreased (18.3 fold) in AML patients compared to controls ($P = 0.03$ and $P < 0.001$, respectively). Both miR-181b and miR-222 were not associated with response to treatment ($P > 0.05$). Also, miR-181b and miR-222 were not differentially expressed in AML patients with M3 compared to non-M3 FAB subtypes ($P > 0.05$). miR-181b and miR-222 are aberrantly expressed in AML patients and their baseline level is not associated with response to treatment.

Keywords Acute myeloid leukemia (AML) · miR-181b · miR-222 · Chemosensitivity

✉ Mani Ramzi
ramzim96@outlook.com

1 Hematology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

2 Shiraz Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

Introduction

Acute myeloid leukemia (AML) is a cancer of blood cells with myeloid origin, characterized by uncontrolled growth of abnormal white blood cells in the bone marrow which interferes with the production of normal blood cells. Although allogeneic hematopoietic stem cell transplantation (HSCT) is a well-established treatment for AML patients, up to 75% of patients develop acute graft-versus-host disease (aGvHD) at the first three months which is still the main cause of morbidity and mortality after allo-HSCT [1]. Until now, no validated diagnostic or predictive markers have been identified for the occurrence of aGvHD.

MicroRNAs are small 18–25 nucleotide non-coding RNAs that target the 3′-untranslated (3′-UTR) regions of specific cellular messenger RNAs (mRNAs) which can modulate the patterns of gene expression [2, 3]. Recently, dysregulated expression of various miRNAs has been reported in hematologic malignancies, especially AML disease which highlight that miRNAs may have prognostic significance in the clinical outcome of AML patients [2–5]. In this regard, miR-181family (a/b/c and d) and miR-222 are among the well-studied miRNAs in AML. Different studies have shown that expression of both miR-222 and miR-181b is dysregulated in AML patients [6–9]. In addition, altered expression of various miRNAs has been shown to be correlated with the presence of aGvHD which proposed that miRNAs might be used as a biomarker for predicting the development of aGvHD [10–12].

In this study, we investigated the expression of miR-181b and miR-222 in newly diagnosed AML patients and also we evaluated for the first time the association of miR-181b and miR-222 with the occurrence of aGvHD and response to therapy.

Patients and Methods

Patients' Criteria

In this cross-sectional study, 80 newly diagnosed adult De novo AML patients were enrolled who admitted to our referral hospital for hematological malignancies during 2016–2017. The AML disease was diagnosed by an oncologist using morphology, cytochemistry, and immunophenotyping. Clinical and laboratory data including French–American–British (FAB) subclass, complete blood count, blast percentage and hemoglobin (Hb) level, were also collected. All patients received standard induction chemotherapy, which consisted of daunorubicin 45 mg/m^2 on days 1 to 3 and cytarabine 100–200 mg/m^2 on days 1 to 7, followed by high doses of a cytarabine-based consolidation phase (Cytarabine 3 g/m^2 every 12 h for 3 days, repeated for 2 to 3 cycles). For M3 patients, arsenic trioxide (0.15 mg/kg/day IV) was used until bone marrow remission occurs plus ATRA (45 mg/m^2/day) in 2 divided doses in addition to standard induction chemotherapy regiment. From all patients, 35 undergone HSCT from related HLA-matched donors and followed for the development of aGvHD. Accordingly, 14 patients developed aGvHD while 31 did not develop aGvHD. aGvHD was graded according to the classic Glucksberg–Seattle criteria and the International Bone Marrow Transplant Registry [13]. From all aGvHD experienced patients, 6 developed low grade (grade I + II) while 8 developed high grade (grade III + IV) of aGvHD. Eighty age-sex matched healthy individuals were enrolled as control group.

This study was approved by the Ethics Committee of Shiraz University of Medical Sciences and written informed consent was taken from all patients included in this study.

Cytogenetic Analysis

Karyotype was analyzed by standard G-banding technique [14]. Chromosomal abnormalities were tested by reverse transcriptase polymerase chain reaction (RT-PCR) for AML1-ETO and CBFB-MYH11. Patients who were negative for these chromosomal abnormalities were considered as CN-AML. Among 80 AML patients, 27 had normal cytogenetic and 53 had abnormal karyotype.

Sample Collection and Ribonucleic Acid Isolation

Five-milliliter peripheral blood was collected in Ethylenediaminetetraacetic acid (EDTA)-containing tubes from each patient at the time of diagnosis prior to chemotherapy treatment and also healthy individuals. The peripheral blood mononuclear cells (PBMCs) were isolated from each patient and controls using Ficoll-hypaque density gradient centrifugation. Total RNA was extracted by TRIZOL reagent (Invitrogen). The quantity of the extracted RNA was evaluated by Nanodrop (Thermo Fisher Scientific, USA). Then, total RNA was converted into cDNA using Prime Script RT Reagent Kit (Takara, Japan) according to the manufacturer's instruction in the T100 thermocycler (Bio-Rad Laboratories, USA) by specific stem-looped designed primers for each miRNAs.

Quantification of the miR-222 and miR-181b mRNAs Expression Level by SYBR Green Real-Time PCR

For the quantitative analysis of miR-222 and miR-181b mRNAs expression level, the SYBR Green Real-Time PCR method was performed using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Japan) and designed primers specific for each miRNA in an iQ5 thermocycler (BioRad Laboratories, USA). U6 gene was used as the internal control. Melt curve analysis was performed to confirm the specificity of reaction at the end of the program. The expression level of the miR-181b and miR-222 mRNAs was normalized to U6 gene. The changes in the relative expression levels of miR-222 and miR-181b mRNAs were calculated by [$2^{-\Delta\Delta Ct}$] method, where $\Delta\Delta Ct$ = [ΔCt (patients)–ΔCt (controls)] and ΔCt = [Ct (sample)–Ct (housekeeping gene)]. All real-time PCR reactions were performed at least in duplicate wells.

Statistical Analysis

Data were analyzed by SPSS software, version 18. The differences in the mean expression level of miR-222 and miR-181b between patients and controls were compared via independent t test. The association between the mean expression of the miR-222 and miR-181b and laboratory data were analyzed by Pearson correlation test. The expression level of miR-222 and miR-181b was compared between patients according to the presence of aGvHD, response to chemotherapy treatment, and FAB subtypes by independent t test. *P* values less than 0.05 were considered as statistically significant.

Results

From 80 newly diagnosed AML patients, 46 (61.5%) were male and 34 (38.5%) were female. The mean age of AML patients was 49 ± 2.4 with a range 20–86 years. Among all AML patients, 28 (35%) had FLT3-ITD mutation. The

demographic data of patients included mean white blood cells (WBC) counts (50,045 ± 10,691), platelet count (54,136 ± 7788), Hb level (g/dL) (8.1 ± 0.33) and lactate dehydrogenase (LDH) level (U/L) (1433 ± 295).

Aberrant miR-181b and miR-222 Expression in AML Patients

The mRNA expression of miR-181b and miR-222 was compared between patients and controls. After the statistical analysis, our results revealed that the expression of miR-181b was significantly higher (4.7 fold) in AML patients than healthy controls (1.4 ± 0.56 vs. 3.7 ± 0.96, $*P = 0.03$). Conversely, miR-222 was significantly decreased (18.3 fold) in AML patients compared to healthy controls (6.9 ± 0.48 vs. 2.7 ± 1.07, $*P < 0.001$).

MiR-181b and miR-222 Expression in HSCT Patients and Development of the aGvHD

The mean expression of miR-181b and miR-222 was compared between patients with and without aGvHD. The results demonstrated that both miR-181b and miR-222 were upregulated in patients developed aGvHD compared to those without aGvHD, however, the difference was not statistically significant (1.48 ± 9.31 vs. 3.47 ± 7.24; $P = 0.483$ for miR-181b and − 4.31 ± 6.01 vs. − 1.18 ± 5.07; $P = 0.107$ for miR-222, respectively). In addition, both miR-181b and miR-222 were overexpressed in HSCT patients experienced high grade (grade III–IV) compared to those patients who developed low grade (grade 0-II) aGvHD, although the difference was not statistically significant (− 4.97 ± 5.92 vs. − 3.43 ± 6.58; $P = 0.654$ for miR-181b and 0.68 ± 9.85 vs. 2.55 ± 9.34; $P = 0.724$ for miR-222, respectively).

MiR-181b and miR-222 Expression in AML Patients and Response to Treatment

The baseline expression level of miR-181b and miR-222 was evaluated in AML patients according to their response to treatment. Our results demonstrated that both miR-181b and miR-222 gene expression levels were increased in AML patients who did not respond to chemotherapy compared to those patients who responded to chemotherapy, albeit the difference was not statistically significant ($P = 0.3$ and $P = 0.7$, respectively).

MiR-181b and miR-222 Expression According to Cytogenetic Status and FAB Groups

Details of cytogenetic information of AML patients are shown in Table 1. The expression level of miR-181b and miR-222 was compared within AML patients based on their cytogenetic abnormalities. Our results showed that the expression level of both miR-181b and miR-222 did not differ between patients with different cytogenetic aberrations ($P > 0.05$).

Since the FAB group of some AML patients was not defined, based on the cytogenetic aberration, we divided AML patients into M3 and non-M3 groups and compared the expression of miR-181b and miR-222 among them. Accordingly, from 53 patients with abnormal cytogenetic, 22 (41.5%) patients were included in M3 and 31 (58.5%) patients in non-M3 groups. Our results demonstrated that the expression level of both miR-181b and miR-222 did not differ between AML patients with M3 and non-M3 FAB subtypes ($P > 0.05$).

Discussion

In this study, we evaluated the expression of the miR-181b and miR-222 in AML patients and healthy controls and also association with response to treatment and development of aGvHD. Our results showed that miR-181b gene expression level was significantly upregulated in AML patients whereas miR-222 gene expression was remarkably decreased in AML patients compared to healthy individuals.

There is accumulating evidence describing the critical role of the miR-181 family in the regulation of hematopoietic cell differentiation including B cells, T cells, natural killer (NK) cells and megakaryocytes [15, 16]. Previously, Cervigne et al. [17] demonstrated that miR-181b may act as an important adaptor between inflammation and malignant transformation.

Using HL-60 cell line, Chen et al. [18] have shown that by targeting mixed lineage kinase 2 (MLK2), miR-181b regulates the myeloid cell proliferation, which is critical in the progression of AML disease. MiR-221/222 are among the most strongly over-expressed miRNAs in AML and had been known as an oncogene in a number of other malignancies [6, 19, 20]. Rommer et al. [6] reported that primary miR-221/222 (pri-miR-221/222) are over-expressed in AML patients at the time of diagnosis which declines to its baseline level in patients who enter the remission phase. Isken et al. [21] showed that miR-221/222 was significantly expressed at a higher level in AML patients compared to healthy controls.

The discrepancy between our result and other studies may be due to different sample size (both patients and controls), the different ethnic group as well as the method used for gene expression analysis.

There are various reports describing that the profile of miRNAs might be used as a biomarker for prediction of

Table 1 AML patients with different cytogenetic status

Cytogenetic results	No. (%)
t(8;21)(q22;q22); RUNX1-RUNX1T1	13 (16.3%)
inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11	12 (15%)
t(15;17)(q22;q12); PML-RARA	22 (27.5%)
t(9;11)(p22;q23); *MLLT3-MLL*	6 (7.5%)
Normal cytogenetic (CN-AML)	27 (33.7%)

aGvHD in patients undergoing HSCT [10–12, 22]. However, we observed that both miR-181b and miR-222 are not associated with development of aGvHD. Therefore, because of the limited number of our patients with aGvHD, increasing patient number will help us to clarify the critical role of miR-181b and miR-222 in the development of aGvHD.

Another finding of our study was that miR-181b and miR-222 was not associated with response to primary induction chemotherapy. Recent studies have shown that chemosensitivity may be affected by aberrant miRNA expression [23]. Butrym et al. [24] explained that AML patients who responded to azacitidine had lower miR-181 expression at diagnosis compared to those non-responder patients. They also demonstrated that the level of miR-181 is inversely correlated with patients' survival after azacitidine therapy as lower miR-181 at the time of diagnosis was associated with longer survival of AML patients [24]. Therefore, according to our findings, further larger population is needed to understand the role of miR-181 and miR-222 in defining response to treatment in our AML patients.

Conclusion

Our study describes that miR-181b and miR-222 are dysregulated in AML patients and their baseline levels are not associated with the development of aGvHD and response to treatment. Longer follow up of our AML patients and also increasing the sample size is needed for understanding the prognostic significance of miR-181b and miR-222 in the clinical outcome of AML patients.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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