Acute myeloid leukemia (AML) is a blood malignancy characterized by the proliferation of aberrant cells in the bone marrow and blood that interfere with normal blood cells. We have investigated whether changes in the level of micro-ribonucleic acid (miR)-19b, miR-17, and miR-25, Wilms’ tumor (WT1), and CCAAT enhancer-binding protein α (CEBPA) genes expression affect disease prognosis and clinical outcome in AML patients.

The expression level of miR-19-b, miR-17, and miR-25, as well as WT1 and CEBPA genes in a group of patients and controls as well as different risk groups (high, intermediate, and favorite risk), M3 versus non-M3, and graft-versus-host disease (GvHD) versus non-GvHD patients were assessed using a quantitative SYBR Green real-time polymerase chain reaction method.
Introduction

Acute myeloid leukemia (AML) is a kind of hematopoietic stem cell disorder characterized by aberrant myeloid cells differentiation and rapid proliferation of immature myeloblasts.1 AML is known to be the most frequent leukemia in adults, accounting for approximately 25% of all leukemia diagnoses.2 The median age at the time of diagnosis is between 66 and 71 years old. In the United States, the annual AML incidence is estimated to be approximately 5 to 6 cases per 100,000 people. Age has a significant impact on the disease incidence, which ranges from 1.3 cases per 100,000 population in patients less than 65 to 12.2 cases per 100,000 population in those over 65.3 Despite decades of research and accomplishments, the pathophysiology of AML is not well understood.4

A combination of environmental risk factors and cytogenetic and genomic abnormalities are involved in disease progression.5 The most frequent cytogenic risk factors and chromosomal aberrations are trisomy chromosome 8, monosomies, or deletions of part or all of chromosomes 5 or 7, the long arm of chromosome 11 [11q], translocations between chromosomes 15 and 17 [t(15;17)], chromosomes 8 and 21 [t(8;21)], and chromosome 16 inversion (inv[16]).6 Furthermore, some AML patients with normal karyotype contain mutations in genes implicated in signal transduction, pro-proliferative pathways, normal hematopoietic differentiation, and epigenetic regulation.7,8

In addition to abnormalities in cytogenetic profile and recurrent genetic profiles, recent studies have suggested that micro-ribonucleic acids (microRNAs [miRs]) have a role in AML posttranscriptional regulation.9 MiRs, which are short single-stranded noncoding RNA species with a length of around 22 nucleotides, are involved in a variety of biological processes including cell proliferation, apoptosis, immune response, tumorigenesis, and hematopoietic lineage differentiation.10 MiRs are primarily involved in the regulation of gene expression at the translational and posttranscriptional levels. MiRs attach to 3 untranslated region of their target messenger RNA (mRNA) and consequently suppress gene transcription via destabilizing and cleavage of target mRNA.11 Abnormal cellular proliferation and differentiation including hematologic malignancies are linked to defective miRs signaling. The miRs as double-edged swords, can either downregulate oncogenes (tumor suppressor miRs) or tumor suppressor genes (oncomiRs).12 During the AML transformation process progenitor cells undergo continuous genetic and epigenetic abnormalities which affect the pathogenesis and clinical outcomes of AML. The miR-17–92 cluster, which encodes six miRs including the miR-17 and miR-19 families, because of its overexpression in several human cancers have been recognized to have oncogenic properties, notably in myeloid malignancies.13–15 MiR-25, which belongs to the miR-106b-25 cluster, has been linked to several malignancies, and might be used as a potential biomarker for AML.16,17 The Wilms’ tumor (WT1) gene has been found to be overexpressed in several leukemias, particularly AML.18–20 The CCAAT enhancer-binding protein α (CEBPA) gene produces a transcription factor that helps myeloid cells differentiate and arrest growing.21 WT1 and CEBPA gene mutations occur mainly in AML.22,23

AML is a greatly heterogeneous disease due to its genetic and epigenetic complexity. Karyotyping, chromosomal banding technique (fluorescence in situ hybridization), and molecular analysis have helped to reveal several mechanisms behind AML progression but there is still a lot to discover. A comprehensive investigation of biological factors that are associated with AML would be valuable in determining individualized treatment, prognosis prediction, and treatment outcome.7,8,24 Chemotherapy, allogenic, and autogenic hematopoietic stem cell transplantation (HSCT), are the most significant therapeutic choices in AML patients. Posttreatment complications such as acute graft-versus-host disease (aGvHD) and the risk of relapse after complete remission have made the disease challenging to treat.25,26 Efforts in understanding the risk factors that lead to the development of aGvHD is beneficial in predicting the safety of HSCT. In the following study, we have investigated changes in the expression pattern of miR-19b, miR-17, miR-25, WT1, and CEBPA genes in AML patients to investigate whether these factors affect the disease prognosis and treatment outcome. Expression of these factors has been analyzed before and after chemotherapy and in patients who have developed aGvHD.

Results When compared with the baseline level at the period of diagnosis before chemotherapy, the expression of miR-19b and miR-17 in AML patients increased significantly after chemotherapy. The level of miR-19b and miR-25 expression in AML patients with M3 and non-M3 French–American–British subgroups differ significantly. MiR-19b and miR-25 expression was elevated in GvHD patients, while miR-19b and miR-25 expression was somewhat decreased in GvHD patients compared with non-GvHD patients, albeit the difference was not statistically significant. Also, patients with different cytogenetic aberrations had similar levels of miR-19-b and miR-25 expression.

Conclusion MiR-19b, miR-17, and miR-25 are aberrantly expressed in AML patients’ peripheral blood leukocytes, which may play a role in the development of acute GvHD following hematopoietic stem cell transplantation.
Table 1 The primers and PCR condition for the miR-222, miR-181b, and GAPDH gene

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5′-3′)</th>
<th>Thermocycling condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT1</td>
<td>Forward CCAGGCTTTGCTGCTGAG</td>
<td>95°C/2 min, 40 cycles of 95°C/30 s, 57.5°C/20 s, and 70°C/30 s</td>
</tr>
<tr>
<td></td>
<td>Reverse GTGGCTCTAAGTGTATGCTG</td>
<td></td>
</tr>
<tr>
<td>CEBPA</td>
<td>Forward GAAGCAGATGATCTCCAT</td>
<td>95°C/2 min, 40 cycles of 95°C/30 s, 8.5°C/20 s, and 70°C/30 s</td>
</tr>
<tr>
<td></td>
<td>Reverse GCCAGATACAGTGTTGATAT</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward GGACTCATGACCATCCTCA</td>
<td>95°C/2 min, 40 cycles of 95°C/30 s, 57.5°C/20 s, and 70°C/30 s</td>
</tr>
<tr>
<td></td>
<td>Reverse CCATGAGGCAGGCGATAT</td>
<td></td>
</tr>
<tr>
<td>miR-17</td>
<td>Forward GGCAAGAGTCTTACAGTC</td>
<td>94°C/2 min, 40 cycles of 94°C/30 s, 60°C/20 s, and 72°C/30 s</td>
</tr>
<tr>
<td></td>
<td>Reverse GTGCAGGGTCCGAGGT</td>
<td></td>
</tr>
<tr>
<td>miR-19b</td>
<td>Forward GTTTGTGTGCAAATCCCATGCA</td>
<td>94°C/2 min, 40 cycles of 94°C/30 s, 60°C/20 s, and 72°C/30 s</td>
</tr>
<tr>
<td></td>
<td>Reverse GTGCAGGGTCCGAGGT</td>
<td></td>
</tr>
<tr>
<td>miR-25</td>
<td>Forward TTAGGCAGGAAGCTTTGGG</td>
<td>94°C/2 min, 40 cycles of 94°C/30 s, 58.5°C/30 s, and 72°C/30 s</td>
</tr>
<tr>
<td></td>
<td>Reverse GTGCAGGGTCCGAGGT</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CEBPA, CCAAT enhancer-binding protein α; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; miR, micro-ribonucleic acid; PCR, polymerase chain reaction; WT1, Wilms’ tumor.

Materials and Methods

Patients’ Criteria
During 2014 to 2018, 110 newly diagnosed adult de novo AML patients participated in this cross-sectional study at Namazi Hospital in Shiraz, Iran. The AML disease was diagnosed by an oncologist using morphology, cytochemistry, and immune phenol typing. Clinical and laboratory data were also obtained, including the French–American–British (FAB) classification, complete blood count, blast percentage, and hemoglobin level. All patients underwent standard induction chemotherapy, which included daunorubicin plus cytarabine; moreover, M3 patients received arsenic trioxide plus ATRA in two separated doses in addition to the standard induction chemotherapy protocol as previously reported. All patients who had HSCT from related human leukocyte antigen-matched donors were divided into two groups: those who had aGvHD and those who had not. The International Bone Marrow Transplant Registry and the standard Glucksberg–Seattle criteria were used to grade aGvHD. All 42 patients had HSCT; 14 developed aGvHD while the remaining 28 did not. From all patients with aGvHD, 11 individuals developed low-grade (grade I + II) aGvHD, while 7 patients developed high-grade (grade III + IV) aGvHD. The Shiraz University of Medical Sciences Ethics Committee authorized this study, and all patients who took part in it gave written informed consent.

Blood Sample Collection and Ribonucleic Acid Isolation
At the time of diagnosis prior to chemotherapy, each patient and healthy individuals, had 5 mL of peripheral blood drawn in ethylenediaminetetraacetic acid-containing tubes. Ficoll-Hypaque density gradient centrifugation was applied to separate peripheral blood mononuclear cells (PBMCs) from each patient and controls. As earlier noted, total RNA was isolated by the TRIzol reagent (Invitrogen) as shown in the manufacturer’s instructions.

SYBR Green Real-Time Polymerase Chain Reaction
The SYBR Green real-time polymerase chain reaction method was applied to quantify the expression levels of miR-19b, miR-17, miR-25, WT1, and CEBPA mRNAs, using SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara, Japan) and designed specific primers for each miRNA in an iQ5 thermocycler (BioRad Laboratories, United States) according to the manufacturer’s instructions. Statistical Analysis
Statistical Package for the Social Sciences software, version 18, was applied to analyze the data. A paired t-test was used to assess changes in the level of miR-19-b, miR-17, miR-25, WT1, and CEBPA mean expression before and after chemotherapy as well as patients according to the presence of aGvHD, response to chemotherapy, cytogenetic aberration, and FAB subtypes. The chi-square test was also used to examine the mean expression level of miR-19-b, miR-17, miR-25, WT1, and CEBPA in laboratory data. Statistical significance was defined as a p-value of less than 0.05.

Results
There were 57 (51.8%) males and 53 (48.2%) females among the 110 newly diagnosed AML patients. AML patients were 38 ± 2.4 years old on average with a range of 20 to 86 years. The demographic and laboratory characteristics of these patients.

Changes in the Expression of miR-19b, miR-17, and miR-25 in AML Patients after Chemotherapy
Before and after chemotherapy, the level of miR-19b, miR-17, and miR-25 mRNA expression were assessed (Fig. 1). After statistical analysis, we found that the expression of miR-19b...
in AML patients following chemotherapy increased significantly (7.4-fold) compared with the time of diagnosis before chemotherapy (p = 0.01).

Furthermore, our findings demonstrated that after chemotherapy, the expression of miR-17 increased significantly (6.06 times) in AML patients in comparison to the period of diagnosis before chemotherapy (p = 0.01). Conversely, miR-25 did not change significantly (decreased 1.1 times) in AML patients following chemotherapy in comparison to its pre-chemotherapy level (p = 0.9).

**Change in WT1 and CEBPA Expression in AML Patients**
Following chemotherapy, the mRNA expression of WT1 and CEBPA was measured and compared with its baseline level at the time of diagnosis before chemotherapy. Our findings demonstrated that following chemotherapy, the CEBPA expression level increased significantly in AML patients compared with AML patients at the time of diagnosis (p < 0.001). Conversely, the expression of WT1 increased nonsignificantly (four times) in AML patients after chemotherapy in comparison to its initial level at the point of diagnosis (p = 0.06).

**Change in miR-19b, miR-17, and miR-25 expression in HSCT Patients with and without aGvHD**
The mean expression of miR-19b, miR-17, and miR-25 was analyzed in patients with and without aGvHD. Patients with aGvHD displayed greater mean expression of miR-19b, miR-17, and miR-25 than those without aGvHD, although the difference was not statistically significant (1.06 ± 3.5 vs. 3.3 ± 1.2; p = 0.1, -0.86 ± 4.8 vs. 5.1 ± 1.2; p = 0.3, -0.21 ± 3.3 vs. 4.9 ± 18.3; p = 0.1, respectively) (Fig. 2). In addition, miR-19b, miR-17, and miR-25 were upregulated in HSCT patients with high-grade (grade III–IV) aGvHD in comparison to those with low-grade (grade 0–II) aGvHD, though the difference was not statistically significant (1.5 ± 1.8 vs. 3.3 ± 1.2; p = 0.1).

**Table 2** Demographic and laboratory information of AML patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC count</td>
<td>37641.22 ± 5325.61</td>
</tr>
<tr>
<td>PLT count</td>
<td>51774.18 ± 6324.25</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>9.35 ± 0.4</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>1433.65 ± 242.70</td>
</tr>
</tbody>
</table>

**Infection type:**

- Pneumonia: 49
- Sepsis: 39
- Fungal: 22

**FAB subtypes:**

- M3: 24 (21.8%)
- Non-M3: 86 (78.1%)

**Abbreviations:** AML, acute myeloid leukemia; FAB, French–American–British; Hb, hemoglobin; LDH, lactate dehydrogenase; PLT, platelet; WBC, white blood cell.

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**Fig. 1** Change in miR-19b, miR-25, and miR-17 expression in acute myeloid leukemia (AML) patients following chemotherapy compared with the time of diagnosis before chemotherapy treatment.

**Fig. 2** The expression level of miR-19b, miR-25, and miR-17 in acute myeloid leukemia (AML) patients developed acute graft-versus-host disease (aGvHD) (positive) compared with those without aGvHD (negative).
Change in WT1 and CEBPA Expression in HSCT Patients with and without aGvHD

The mean WT1 and CEBPA expression of patients with and without aGvHD was analyzed. The findings demonstrated that patients who experienced aGvHD had a higher mean expression of WT1 and CEBPA compared with those who did not. However, only CEBPA expression showed a statistically significant increase (-3.1 ± 3.2 vs. 3.3 ± 1.01; p = 0.04 for CEBPA and -2.3 ± 3.7 vs. 2.8 ± 1.2; p = 0.14 for WT1, respectively).

WT1 and CEBPA were also upregulated in HSCT patients who developed high-grade (grade III–IV) aGvHD in comparison to those who possessed low-grade (grade 0–II) aGvHD, though the difference was not statistically significant (0.9 ± 1.7 vs. -4.5 ± 10.7; p = 0.5 for WT1 and -0.5 ± 1.6 vs. -8.6 ± 7.7; p = 0.2 for CEBPA, respectively).

Expression of miR-19b, miR-25, miR-17, WT1, and CEBPA in AML Patients based on Their Response to Chemotherapy

The level of miR-19b, miR-25, miR-17, WT1, and CEBPA expression at baseline were assessed in AML patients based on their response to chemotherapy. Our analysis showed that the levels of all miR-19b, miR-25, miR-17, WT1, and CEBPA genes expression levels were higher in AML patients who did not react to chemotherapy than in those who did, while the differences were not statistically significant (p = 0.5, p = 0.6, p = 0.4, p = 0.6, and p = 0.08, respectively).

Expression of miR-19b, miR-25, miR-17, WT1, and CEBPA based on Cytogenetic Status and FAB Groups

Table 3 shows the cytogenetic characteristics of AML patients in detail. FLT3-ITD mutations were found in 33 (30.5%) of all AML cases. AML patients were divided into three groups based on the general genetic risk stratification: favorable, intermediate, and high-risk. As a result, 34 patients were assigned to the high-risk group, 54 to the intermediate risk group, and the remaining 22 to the favorable risk group. Then, within each risk stratification group, the mean expression level of miR-19b, miR-25, miR-17, WT1, and CEBPA were compared. Based on their cytogenetic abnormalities, the expression level of miR-19b, miR-25, miR-17, WT1, and CEBPA were compared in AML patients.

Table 3: Acute myeloid leukemia with recurrent cytogenetic abnormalities

<table>
<thead>
<tr>
<th>Cytogenetic abnormalities</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(8;21)(q22;q22); RUNX1-RUNX1T1</td>
<td>19 (15.5)</td>
</tr>
<tr>
<td>inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11</td>
<td>15 (12.7)</td>
</tr>
<tr>
<td>t(15;17)(q22;q12); PML-RARA</td>
<td>24 (19.1)</td>
</tr>
<tr>
<td>t(9;11)(p22;q23); MLLT3-MLL</td>
<td>11 (9.1)</td>
</tr>
</tbody>
</table>

According to our findings, the expression level of miR-19b and miR-25 did not change between patients with different cytogenetic abnormalities (p > 0.05). Because the FAB group from certain AML patients was determined, we classified AML patients into M3 and non-M3 divisions and investigated the expression of miR-19b, miR-25, miR-17, WT1, and CEBPA among them. Our findings demonstrated that the miR-19b and miR-25 expression level differ significantly between M3 and non-M3 FAB categories of AML patients (4.9 ± 1.03 vs. 1.7 ± 0.89; p = 0.02, 5.9 ± 1.2 vs. 1.5 ± 1.1; p = 0.01, respectively). Also, our findings revealed that the level of miR-17, WT1, and CEBPA expression in M3 and non-M3 FAB categories of AML patients did not change significantly (p > 0.05).

MiR-19b and miR-25 Expression according to White Blood Cell Count and Gender

A significant association between miR-19b and miR-17 expression and white blood cell count in AML patients was seen (p = 0.01 and p = 0.04, respectively). In addition, female patients had significantly greater level of miR-25 and WT1 expression than male patients (p = 0.01 and p = 0.01, respectively). There was also a positive association between gender and CEBPA expression in patients with AML (p = 0.02).

Discussion

A combination of genetic abnormalities, gene deregulations and mutations, and chromosomal rearrangements, play a crucial role in AML development. The aberrant expression profile of miRNAs can seriously affect cell proliferation, survival, and hematopoietic differentiation. High clinical heterogeneity of the disease has made the treatment decision challenging, so different therapeutic interventions are required.

The WT1 gene encodes a zinc finger transcription factor and is found on the short arm of chromosome 11 (11p13). WT1 expression is observed in CD34+ bone marrow-derived cells in normal hematopoiesis. In addition, WT1 interacts with a variety of proteins, it binds to P53 and thus prevents apoptosis. WT1 also interacts with signal transducer and activator of transcription 3 which results in enhanced cell proliferation. WT1 overexpression has been informed in 70 to 100% of AML patients and mutation of the WT1 gene has also been demonstrated in 10 to 15% of AML cases. As a result, it can be a tumor suppressor gene or an oncogene. Recent studies have shed light on the undeniable role of WT1 on disease prognosis and treatment outcome. The level of WT1 expression is used as a predictive marker in disease relapse. It is also used to evaluate minimal residual disease after initial chemotherapy. CEBPA is a leucine zipper transcription factor that plays an important role in the hematopoiesis process and cell-cycle arrest, and the inhibition of self-renewal and myeloid differentiation.
expression is upregulated during the commitment of multipotent precursors to the myeloid lineage. CEBPA gene mutation is been informed in 5 to 14% of AML patients. In 2019, Gholami et al. showed that CEBPA expression is raised in AML patients and higher CEBPA expression plays a significant role in AML pathogenesis. Many miRNAs are known to be CEBPA downstream effectors. For example, miR-223 activation via CEBPA plays a key role in neutrophil differentiation and function. It has also been reported that in AML patients impaire CEBPA function leads to reduced expression of miR-29b which is a tumor suppressor gene. Impaired CEBPA-miR-182 balance also is related to adverse prognosis in AML patients. According to our results, CEBPA gene expression is higher in patients; however, the difference is not statistically significant. Patients who had higher CEBPA expression before chemotherapy did not respond to chemotherapy. Our data also revealed that CEBPA expression significantly increased after chemotherapy compared with its value before treatment. CEBPA expression is significantly related to aGvHD development. The miR-25 belongs to the miR-106b-25 cluster, which is found on chromosome 7q22.1. The miR-25 is known to be involved in several kinds of solid cancers. In 2014, Xiong et al. investigated the expression pattern of miR-25 in three leukemic cell lines (HL-60, THP-1, and K562) which revealed elevated expression of miR-25 compared with normal cells. Niu et al. investigated the role of miR-25 in chemotherapy and also HSC outcome. According to this study higher expression of miR-25 in AML patients is related to favorable treatment outcomes. In a cohort study of 122 newly diagnosed AML patients with predominantly intermediate and poor-risk cytogenetics by Garzon et al., miR-20a, miR-25, miR-191, miR-199a, and miR-199b were shown to be overexpressed. In the present study, our data revealed that miR-25 expression is significantly elevated in AML patients. The expression level of miR-25 was also compared in patients before and after chemotherapy and in those patients who suffered from aGvHD. The role of miR-25 in response to chemotherapy was also investigated. Although it was not statistically significant, those who did not respond to chemotherapy had a higher expression of miR-25. This may be due to our relatively small sample size. To comprehensively understand the biological mechanism underlying miR-25 the target genes of miR-25 is valuable in revealing the leuke-mogenic role of miR-25. MYH9 is identified to be a direct target of miR-25. High MYH9 expression is involved in resistance to chemotherapy and may be indicative of poor clinical outcomes in AML patients. The miR-25 expression is negatively related to HOXA4, HOXB8 gene clusters. HoxB4 is involved in hematopoietic stem cell renewal. On the other side, HOXB9 plays an important role in hematopoietic stem cell expansion. CEBPA is involved in HOXB9 mediated leukemogenesis. The miR-17 and miR-19b are members of the miR-17–92 cluster, located on chromosome 13 (13q31.3), and was confirmed to have oncogenic properties. Recent studies revealed the elevated expression of miR-19b in patients with de novo AML which matched our findings.

Conclusion
Despite the small sample size, our findings suggest that miR-19b, miR-17, and miR-25 were aberrantly expressed in PBMCs of AML patients, especially in those with M3 FAB subtypes. Other studies regarding mRNAs and coinhibitory molecules revealed an increase in the percentage of AML patients, notably those who received HSCT and suffered from aGvHD, that were used as a targeted therapy in line with the standard conventional chemotherapy for minimizing the risk of aGvHD subsequent to HSCT.

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Conflict of Interest
None declared.

Acknowledgments
None.

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