ABSTRACT

Acute leukemia (AL) is one of the most common hematologic malignancies in children. Acute leukemia characterized by several genetics changes which could affect patient’s prognosis. Chromosomal rearrangements involving MLL gene have been associated with dismal outcome in acute leukemias. The present study projected to detect the six most frequent MLL fusion transcripts in Iranian childhood acute leukemia patients. Peripheral blood (PB) and bone marrow (BM) samples of 115 pediatric (one month to 15 years) newly diagnosed AL patients were subjected for molecular analysis. Written consent was obtained from the patient’s parents. Paired multiplex reverse-transcriptase polymerase chain reaction (PMRT-PCR) technique was performed for molecular analysis. Only 16.4% of cases were AML. Disease recurrence was seen in 16.4%. Regard to vital status 82.6% of cases was alive. Obtained results revealed 2.6% MLL rearrangement positive in samples. MLL rearrangement negative had longer OS. Quantitative RT-PCR assay of MLL revealed a down regulation in 44% patients. Data analysis shows no correlation between MLL gene expression and CBC indices. In relation to obtained data it concluded that multiplex PCR as a sensitive molecular technique is willing to come in routine molecular labs.

KEYWORDS: MLL, childhood acute leukemia, pair multiplex RT-PCR, Quantitative RT-PCR, Gene rearrangement

INTRODUCTION

Leukemias are common hematological cancers worldwide with highprevalence in children. Risk factors and prognosis are differing between children and adults leukemias. They comprise a group of clinico-biological diseases which are characterized by several genetic alterations. Acute leukemia (AL) is the most frequent childhood cancer divided into acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML). It has been considered that about 80% of leukemias are lymphocytic in origin. Genetic alterations affecting the cell cycle process leading to diverse collection of abnormalities especially in stem cells. According to numerous studies, a wide variety of genetic changes underlies disruption of proliferation and differentiation of cells originating from hematopoietic stem cells. The type of genetic aberration is extremely important prognostic factor in AL. Chromosomal abnormalities involving mixed-lineage leukemia (MLL gene on chromosome band 11q23 are recurrent events in AL and strongly associated with survival of patients. Different MLL fusion partners offer a possible role in the leukemogenesis. Regarding the oncogenic activity of the most MLL partner proteins, they have potency for transformation in unique gene expression profiles. It has been determined that MLL rearrangements are present in ~10% of human leukemias. MLL translocations account for up to 80% of infant leukemia, but low extent in older children. Identification of the specific chimeric gene primarily is more importance for diagnosis, treatment decision plan and highlighted as extraordinary markers in minimal residual disease monitoring. According to the literature there are several methods such as Chromosome banding analyses and molecular techniques for detection of 11q23/MLL.
abnormalities. Conventional cytogenetics testing is difficult and dependent on good chromosome morphology. Interphase fluorescence in situ hybridization (FISH), Southern blot and split-signal analysis of the MLL gene do not provide an informative diagnostic result. The present multi-center study aimed to explore the occurrence of MLL rearrangements among children with AL.

MATERIAL AND METHODS

Ethics statement
The current study was carried out under supervision of TUMS ethics committee (Ethics code: IR.TUMS.REC.1394.1792) and written informed agreement was obtained from the patient’s parents before sample collection. The ethics committee’s approval included a review of the consent procedure. All patients' information was an onynized.

Sampling
Peripheral blood (PB) and bone marrow (BM) samples were obtained from 115 children under 15 years old who diagnosed with acute leukemia referred to Children’s Medical Center, Imam Khomeini Hospital Complex- Valiasr Hospital and Mofid Children's Hospital, Tehran, Iran. Initially, acute leukemia diagnosis was based on morphology, cytochemistry and immune phenotypic assessments. Mononuclear cells were isolated from PB/BM samples by Ficoll-Hypaque density centrifugation washed twice with normal saline and 10⁸ cells suspended in 1 mL of Trizol reagent (Gibco-BRL, Life Technologies) and stored at −75°C until test. Total RNA was isolated from the thawed cells by Trizol method (Invitrogen, USA) following the manufacturer’s recommendations. The quality and quantity of total RNA were verified by gel electrophoresis and optical density reading with a Nano Drop (ThermoScientific2000, USA). Then, 5 μg of total RNA was used to synthesize complementary DNA (cDNA) fragments using random sequence hexamer primers and OligodT with a cDNA synthesis system kit (yektaTaghizAzma, Tehran, Iran), according to the manufacturer’s instruction. The cDNA and was stored at -20°C until used for PCR amplification. cDNA synthesized from 115 samples amplified for ABL housekeeping gene to check the integrity of cDNA.

Molecular diagnosis
Paired multiplex reverse-transcriptase polymerase chain reaction (PMRT-PCR) assays were performed according to the criteria described by Andersson et al. to identify the six commonest MLL transcription partner genes generated t(4;11)(q21;q23) [MLL/AF4], t(6;11)(q27;q23) [MLL/AF6], t(9;11)(p21–22;q23)[MLL/AF9], t(10;11)(p11–13;q23)[MLL/AF10], t(11;19) (q23;p13.1) [MLL/ELL], and t(11;19)(q23;p13.3) [MLL/ENL]. For this purpose 2μL of cDNA were used for the subsequent PMRT-PCR assay, as described. We first used the known diagnosed positive samples to optimize the multiplex RT-PCR assay.

STATISTICS
Data were collected from the medical records. The results were summarized and carried out in the form of mean, range, percentage and standard deviation as descriptive statistics. Descriptive statistics and statistical comparison were performed applying the SPSS 22 statistical software and prism 5. Comparison was done regarding clinical data using the Chi-Square test. Independent T-test and ANOVA test were used for laboratory data. The survival rates were estimated by applying the Kaplan-Meier method. A p-value <0.05 was considered as statistically significant. Overall survival probability (OS), and event free survival probability (EFS) were defined as previously.
patients relapsed during therapy; Statistical analysis determined an event-free survival rate of 69.8% and Overall survival (OS) 82.5% in AL samples. OS was shorter in cases with MLL rearrangement (p<0.05).

### Table 1
**Patients’ characteristics**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Subgroups</th>
<th>ALL</th>
<th>AML</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age groups</td>
<td>&lt;1</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1-10</td>
<td>80</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>&gt;10</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>52</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>44</td>
<td>10</td>
</tr>
<tr>
<td>Follow up duration</td>
<td>Length(month)</td>
<td>9(&lt;1-25)</td>
<td>7(1-17)</td>
</tr>
<tr>
<td></td>
<td>CR after induction</td>
<td>86</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Induction failure(refractory)</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Relapse</td>
<td>0</td>
<td>81</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>≥ 2</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Immunophenotype</td>
<td>B-ALL</td>
<td>92</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>T-ALL</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>AML</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>Outcome</td>
<td>Alive</td>
<td>80</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Dead</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>Risk factors</td>
<td>Age ≥ 10</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Age ≤ 1</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

### Table 2
**Clinical characteristics and molecular rearrangements of the two acute leukemias with 11q23 rearrangements**

<table>
<thead>
<tr>
<th>Case No</th>
<th>Age/Sex (years)</th>
<th>Diagnosis</th>
<th>11q23abnormality</th>
<th>Partner gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3/M</td>
<td>AML</td>
<td>t(4;11)(q21;q23)</td>
<td>AF4</td>
</tr>
<tr>
<td>2</td>
<td>7/F</td>
<td>ALL</td>
<td>t(4;11)(q21;q23)</td>
<td>AF4</td>
</tr>
<tr>
<td>3</td>
<td>4/M</td>
<td>ALL</td>
<td>t(11;19)(q23;p13.3)</td>
<td>ENL</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Leukemia is one of the most prevalent malignancies with an increasing prevalence in Iran\(^7,16\). The exact frequency of these types of childhood blood cancers is due to absence of a developed cancer registry system\(^13\). Despite the improvements in treating childhood cancer, leukaemia remains with high mortality rate in children\(^14\). It is well known that, leukemia is highly expensive for diagnosis and therapy\(^16,17\). Diverse molecular genetic alterations considerably influencing prognosis and treatment decisions of childhood with acute leukemia. On the other hand, poor condition leading to difficulty in timely accurate diagnosis and effective treatment in Iran\(^7\). Particularly unfavorable survival rates are found in patients with abnormalities of the MLL gene\(^3\). It is well known that leukemia with 11q23/MLL rearrangements has high risk group and must undergo intensified treatment\(^5\). We aimed to reveal MLL gene rearrangements among childhood with acute leukemia patients and get insight about their conditions. In the present study, we identified three cases with MLL gene abnormality using RT-PCR as an accepted ideal molecular technique\(^12,18,19\). Analysis of patient’s medical records showed 42%and 36% availability of molecular test results and cytogenetic analysis, respectively. Among them only MLL-AF4 fusion gene was investigated. Base on review of literatures the six most common cover more than 90% of all MLL abnormalities with comprises worse prognosis in involved patients\(^10,18\). Overall frequency of MLL rearrangements were 2.6% in our samples which is in line with another reports\(^20,21\). In cases of too small alteration, conventional cytogenetic may miss to identify specific changes. Many researchers reported that RT-PCR to be more sensitive than conventional
cytogenetics\(^9\). In other words, accurate and timely detection of those frequent MLL fusion gene is extremely important to allow appropriate treatment stratification as well as risk assessment; ultimately, therapeutic regime can be personalized\(^3\). Several scientists were suggested multiplex PCR could be uses routinely for convenient molecular analysis for hybrid transcripts identification in leukemia patients. The other benefits of this methods are include relative reliability, easy and cost effective makes it an enable sensitive tool for detecting respective MLL fusion genes which are helpful for clinicians to proper treatment decision\(^9,18\). Findings of the present work were revealed frequency of 82.6% ALL with predominant B lineage which is in agreement with other reports around the word\(^22,23\). According to numerous studies MLL gene rearrangements are account for 5-10% of genetic alteration of childhood acute leukemiacases\(^24-27\). Our results showed 2.6% dropped in this range. At 620 days, overall survival and event-free survival rates were 82.6% and 69.5%, respectively which are in compatible with another study other reports\(^28\). In confirm the previous studies OS in our MLL rearranged patients were significantly lower than others (P<0.05)\(^27-31\). Relapse incidence rate was 1.6%. Induction failure was seen in 11.3% of samples. Two of 3 MLL-r patients achieved complete remission and one died\(^25,27,31\). Of all 66.5% of cases were achieved complete remission. It was in compatible with some Asian neighbors countries15and considerably improved as compared to other Iranain reports\(^33,34\).We concluded molecular methods such as multiplex PCR which are sensitive to detect common chromosomal abrasion helpfully could come to routine practice as a reliable way to cover missing diagnosed.

**CONFLICT OF INTEREST**

Conflict of interest declared none.

**REFERENCES**


