RESEARCH ARTICLE

Multiplex RT-PCR Assay for Detection of Common Fusion Transcripts in Acute Lymphoblastic Leukemia and Chronic Myeloid Leukemia Cases

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Abstract

Background: Acute lymphoblastic leukemia (ALL) is a heterogeneous disease which requires a risk-stratified approach for appropriate treatment. Specific chromosomal translocations within leukemic blasts are important prognostic factors that allow identification of relevant subgroups. In this study, we developed a multiplex RT-PCR assay for detection of the 4 most frequent translocations in ALL (BCR-ABL, TEL-AML1, MLL-AF4, and E2A-PBX1).

Materials and Methods: A total of 214 diagnosed ALL samples from both adult and pediatric ALL and 14 cases of CML patients (154 bone marrow and 74 peripheral blood samples) were assessed for specific chromosomal translocations by cytogenetic and multiplex RT-PCR assays.

Results: The results showed that 46 cases of ALL and CML (20.2%) contained the fusion transcripts. Within the positive ALL patients, the most prevalent cryptic translocation observed was mBCR-ABL (p190) at 8.41%. In addition, other genetic rearrangements detected by the multiplex PCR were 4.21% TEL-AML1 and 2.34% E2A-PBX1, whereas MLL-AF4 exhibited negative results in all tested samples. Moreover, MBCR-ABL was detected in all 14 CML samples. In 16 samples of normal karyotype ALL (n=9), ALL with no cytogenic result (n=4) and CML with no Philadelphia chromosome (n=3), fusion transcripts were detected.

Conclusions: Multiplex RT-PCR provides a rapid, simple and highly sensitive method to detect fusion transcripts for prognostic and risk stratification of ALL and CML patients.

Keywords: Acute lymphoblastic leukemia - chronic myeloid leukemia - multiplex RT-PCR - risk stratification

Introduction

Acute lymphoblastic leukemia (ALL) is a heterogeneous group of clonal hematopoietic stem cell malignancies with variable clinical represents, cell morphologies, immunophenotypes, genetic alterations, prognosis, disease progressions, as well as treatment outcomes (Piwkham et al., 2015). The recent revision of the World Health Organization (WHO) 2008 classification of myeloid neoplasms and acute leukemia has been categorized chronic myeloid leukemia (CML), acute myeloid leukemia (AML), and acute lymphoblastic leukemia (ALL) based on the representation of specific balanced chromosomal translocations (Vardiman et al., 2009). Conventional karyotyping could identify common chromosomal translocations in leukemia which are recognized as disease etiology, reflecting on risk-stratification and providing applicable therapeutic strategies including: AML is associated with t(8;21) (q22;q22), inv(16)(p13.1q22) or t(16;16)(p13.1;q22), t(15;17)(q22;q12), t(9;11)(p22;q23), t(6;9)(p23;q34), inv(3)(q21q26.2) or t(3;3)(q21;q26.2), t(1;22)(p13;q13); ALL is mostly involved t(9;22)(q34;q11.2), t(1;19)(q23;p13.3) and CML is characterized by the identification of t(9;22) (q34;q11.2) (Philadelphia chromosome) (Look, 1997; Awan et al., 2012; Xiong et al., 2013; Ye et al., 2014; Tahira et al., 2015). Although complete cytogenetic studies by karyotyping of bone marrow sample obtained from the patient has been proved to be the gold standard method (especially in CML) and proposed for an initial assessment tool to determine the baseline of patient karyotype, the test has several disadvantages such as required fresh specimen, labor intensive, time consuming, technically difficult, requires high skilled personnel, facing with contamination and culture failure, and cannot detect some minute genetic alterations (Shaikh et al., 2014). Moreover, karyotyping could not perform in treated patients who show complete

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cytogenetic response (CyR) after induction therapies (Goldman, 2005). In the past decade, fluorescent in situ hybridization (FISH) technique using fluorescent-labeled probes specific to unique chromosomal translocation has been shown superior advantages over conventional karyotyping including high sensitivity and specificity, can be performed on both dividing and non-dividing cells (metaphase/interphase cells), fast, can analyze many cells in the same time, and could be a monitoring test for the detection of minimal residual disease (MRD) in leukemia (Krauter et al., 1998; Elmaagacli, 2007; Park et al., 2008; Testoni et al., 2009; Mazloumi et al., 2012). Nevertheless, several limitations of FISH have been observed such as not a screening test, requires specific probes, low resolution in particular probe set, and less sensitivity than the PCR-based technology. At present, the reverse transcriptase-PCR assay (RT-PCR) has been shown to be a powerful sensitive, specific, and rapid test for detection of genetic alterations including recurrent chromosomal translocations in leukemia (Scurto et al., 1998; van Dongen et al., 1999; Harrison, 2000; Liang et al., 2002; Elia et al., 2003; Yang et al., 2010). Moreover, several studies demonstrated that RT-PCR has greater sensitivity than conventional karyotyping and equivalent to FISH for detecting and monitoring of MRD in several types of leukemia (Colleoni et al., 2000; Sarriera et al., 2001; Raanani et al., 2004). Furthermore, in almost last two decades, several multiple RT-PCR systems have been developed for routinely used as a screening test for revealing recurrent genetic alterations in leukemia based on its ability to simultaneously detect multiple genetic aberrant/chromosomal translocations (van Dongen et al., 1999; Salto-Tellez et al., 2003; Dunlap et al., 2012; Xiong et al., 2013). In this report, we described our routine multiplex RT-PCR assay for detecting and screening recurrent translocations in CML and ALL patients.

Materials and Methods

Patient samples

A total of 214 diagnosed ALL samples from both adult and pediatric ALL and 14 cases of CML patients (154 bone marrow and 74 peripheral blood samples) who registered for leukemia diagnosis at Department of Pathology, Ramathibodi hospital, Mahidol University, Bangkok, Thailand during January 2010 to April 2015 were collected and subjected to this study. Diagnostic criteria was based on the representation of hematologic findings, histology, the presence of leukemic blast (lymphoid lineage) and immunophenotypes. Complete cytogenetic study according to the recently revised WHO 2008 classification of myeloid neoplasms and acute leukemia (Vardiman et al., 2009) was done.

Cytogenetic study

Complete cytogenetic study or karyotyping was performed at Human Genetic Laboratory, Department of Pathology, Ramathibodi hospital using G-banding technique after short term culture without mitogen activation. On-screen karyotyping was performed on 20-30 metaphases using Ikaros software, MetaSystems, Germany. Chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature (ISCN, 2013) (Shaffer et al., 2013).

RNA extraction, RNA quantification, and cDNA synthesis

Total RNA was extracted from peripheral blood and bone marrow samples using QIAamp RNA Blood Mini Kit (Qiagen) according to the manufacturer’s recommendations. RNA concentration and qualification were determined by NanoDrop 2000 (Thermo Scientific, MA, USA) spectrophotometer. One nanogram of RNA was transcribed to complementary DNA (cDNA) using SuperScript® VILO™ CDNA synthesis kit (Life Technologies, USA) according to the manufacturer’s recommendations.

Multiplex PCR

Single-round multiplex PCR reaction specific for amplification of four common chromosomal translocations in ALL (TEL-AML1, MLL-AF4, E2A-PBX1, and BCR-ABL; p190), the additionally BCR-ABL; p210 in CML, and internal control HPRT (hypoxanthine ribosyltransferase) genes was performed to amplify cDNA generated from RNA isolated from ALL patients. Primers details are described in Table 1. Optimal multiplex PCR condition was following: 95°C for 10 minutes, 35 cycles of 95°C for 30 seconds, 65°C for 30 seconds, 72°C for 30 seconds, and final extension at 72°C for 10 minutes. PCR was held at 4°C until continue to further steps. 10 µl of PCR products were detected by automated electrophoresis, QIAxcel Advanced system (QIAGEN, Germany).

Quality Control

Multiplex RT-PCR assay was performed using leukemia cell line as positive controls for specific chromosomal translocations (K562 cell line with MBCR-ABL p210 and SUP-B15 cell line with mBCR-ABL p190). For the other three translocations, cDNA from patients positive for cryptic translocations by conventional cytogenetics including, t(12;21)(p13;q22) and t(1;19) (q23;p13.3) was used as positive controls for TEL-AML1 and E2A-PBX1, respectively. For t(4;11)(q21;q23) positive control, plasmid DNA carrying MLL-AF4 was used.

Results

Multiplex PCR assay potentially used as a screening and monitoring test for ALL and CML

A total of 228 samples (154 bone marrow aspirate and 74 peripheral blood samples) collected from ALL (n = 214) and CML (n = 14) patients were analyzed using conventional karyotyping and subsequently examined by the established multiplex PCR. Multiplex PCR following with gel electrophoresis was performed as described in material and method section. The interpretation of the finding was shown in figure 1. All samples were positive for a housekeeping gene (HPRT) by the multiplex PCR (except lane 4 which is a plasmid control DNA of MLL-AF4). The established multiplex PCR was able to detect...
BCR-ABL fusion in all 14 samples collected from CML patients. Interestingly, this method was able to detect BCR-ABL fusion transcripts in 2 treated CML patients who showed negative result for Philadelphia chromosome by conventional genetic study (No.3 and 23 in Table 2). These results indicated that our proposed multiplex PCR could be potentially used as a routine screening and monitoring test for CML. In diagnosed ALL samples, 32 samples were positive for the generated multiplex PCR analysis (15.00 %) whereas 182 samples (85.00 %) showed negative results. Within the positive group, the most prevalent cryptic translocation observed in our tested samples was the mBCR-ABL (p190) with 8.41 % (18/214). Other genetic rearrangements detected by the established multiplex PCR in ALL were following, TEL-AML1 with 4.21% (9/214), E2A-PBX1 with 2.34% (5/214), whereas MLL-AF4 exhibited negative result in all tested samples (Figure 2). Furthermore, the generated multiplex PCR assay was able to identify those fusion transcripts (except MLL-AF4) in 14 samples which could not be detected by the routinely conventional cytogenetic analysis (Table 2). To gather, our established multiplex PCR assay could be potentially used as a combined test with conventional cytogenetic study for screening and detecting common cryptic chromosomal translocations as well as a monitoring test for ALL and CML.

PCR sequencing revealed genetic breakpoint of TEL-AML1 and E2A-PBX1 sharing between individual ALL patients

To further confirm the positive results by our proposed multiplex PCR and to investigate the structure of recurrent chromosomal translocations in each patient, we performed PCR sequencing assay specific to each fusion gene including TEL-AML1 and E2A-PBX1. We were able to sequence 3 of 9 cDNA samples from TEL-AML1 positive patients (33.33 %) and 3 of 5 cDNA samples from patients with E2A-PBX1 (60.0 %), respectively. We observed that

Table 1. PCR Primers for Multiplex PCR Amplification of Common Translocations in ALL and CML

<table>
<thead>
<tr>
<th>Chromosomal translocation</th>
<th>Fusion gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Reference sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(12;21)(p13;q22)</td>
<td>TEL-AML1</td>
<td>CGAGGACGGGCTGCATAAGCGCCTGCTCATCTTTGNM_001754.4</td>
<td>NM_001987.4</td>
</tr>
<tr>
<td>t(1;19)(q23;p13.3)</td>
<td>E2A-PBX1</td>
<td>GCCCTCCCAGCTCTACAGTTCTGATTTCCTCCAGCTCCGTATGNNM_00120496.1</td>
<td>NM_003200.3</td>
</tr>
<tr>
<td>t(4;11)(q21;q23)</td>
<td>MLL-AF4</td>
<td>CCGCCAAGTATCCTCTGTAACAACTACCTCAGTATTCTCGAAGCCTACAGT</td>
<td>NM_00119704.1</td>
</tr>
<tr>
<td>t(9;22)(q34;q11.2)</td>
<td>mBCR-ABL</td>
<td>CGCAAGACGGCCAGATACCTCGAAGCCTGCTCATCTTTGNM_001754.4</td>
<td>NM_021574.2</td>
</tr>
<tr>
<td>t(9;22)(q34;q11.2)</td>
<td>MBCR-ABL</td>
<td>AAGAACCTGTCAGGACAGAGAAGAACCTGTCAGGACAGGTTCAAGAAAGACAGAT</td>
<td>NM_004327.3</td>
</tr>
<tr>
<td>House-keeping (control) gene</td>
<td>HPRT</td>
<td>TCAAACAAACATCGCCTTTAAAAAGCGCTGAAGTATCTCGAAGCTAGTAGTAA</td>
<td>NM_000194.2</td>
</tr>
</tbody>
</table>
### Table 2. Data Summary of Samples Positive for the Established Multiplex PCR

<table>
<thead>
<tr>
<th>No</th>
<th>Sample</th>
<th>Age (Year)</th>
<th>Sex</th>
<th>Karyotyping</th>
<th>Diagnosis</th>
<th>Multiplex PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PB</td>
<td>25</td>
<td>M</td>
<td>46.XX,t(9;22)(q34;q11.2) [6]/46.XY [25]</td>
<td>CML</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>2</td>
<td>PB</td>
<td>32</td>
<td>F</td>
<td>46.XX,t(9;22)(q34;q11.2) [6]/46.XY [25]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>3</td>
<td>BM</td>
<td>26</td>
<td>M</td>
<td>46.XX [30]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>4</td>
<td>BM</td>
<td>42</td>
<td>F</td>
<td>46.XX,t(9;22)(q34;q11.2) [6]/46.XY [25]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>5</td>
<td>BM</td>
<td>14</td>
<td>M</td>
<td>50.XY,+t(5;9)+2mar2[2]/46.XY [33]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>6</td>
<td>BM</td>
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<td>F</td>
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<td>ALL</td>
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</tr>
<tr>
<td>7</td>
<td>PB</td>
<td>ND</td>
<td>M</td>
<td>46.XX [20]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>8</td>
<td>PB</td>
<td>80</td>
<td>F</td>
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<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>9</td>
<td>PB</td>
<td>53</td>
<td>M</td>
<td>45.XY,t(9;22)(q34;q11.2) [6]/46.XY [25]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>10</td>
<td>PB</td>
<td>43</td>
<td>F</td>
<td>46.XX [20]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>11</td>
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<td>23</td>
<td>M</td>
<td>46.XX,t(9;22)(q34;q11.2) [6]/46.XY [25]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>12</td>
<td>PB</td>
<td>43</td>
<td>F</td>
<td>46.XX [20]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>13</td>
<td>BM</td>
<td>43</td>
<td>M</td>
<td>46.XX [30]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>14</td>
<td>BM</td>
<td>46</td>
<td>M</td>
<td>46.XX [30]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>15</td>
<td>BM</td>
<td>43</td>
<td>F</td>
<td>46.XX,t(9;22)(q34;q11.2) [6]/46.XY [25]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>16</td>
<td>BM</td>
<td>42</td>
<td>M</td>
<td>46.XX [30]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>17</td>
<td>PB</td>
<td>43</td>
<td>M</td>
<td>46.XX [30]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>18</td>
<td>BM</td>
<td>54</td>
<td>M</td>
<td>46.XX [30]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>19</td>
<td>BM</td>
<td>54</td>
<td>M</td>
<td>46.XX [30]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>20</td>
<td>BM</td>
<td>43</td>
<td>F</td>
<td>46.XX [30]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>21</td>
<td>BM</td>
<td>52</td>
<td>M</td>
<td>46.XX [30]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>22</td>
<td>BM</td>
<td>49</td>
<td>M</td>
<td>46.XX [30]</td>
<td>ALL</td>
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</tr>
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<td>23</td>
<td>BM</td>
<td>11</td>
<td>F</td>
<td>46.XX,t(9;22)(q34;q11.2) [6]/46.XY [25]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
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<tr>
<td>24</td>
<td>BM</td>
<td>12</td>
<td>F</td>
<td>46.XX,t(9;22)(q34;q11.2) [6]/46.XY [25]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
<tr>
<td>25</td>
<td>PB</td>
<td>42</td>
<td>F</td>
<td>46.XX [20]</td>
<td>ALL</td>
<td>mBCR-ABL (p190)</td>
</tr>
</tbody>
</table>
TEL-AML1 gene rearrangements were conserved between individual patients. This common breakpoint was sparing through exon 5 of TEL and fused to the end of exon 5 of AML1 (Figure 3). Likewise, all patients with E2A-PBX1 positive showed similar breakpoint structures which fused exon 14 of E2A to exon 2 of PBX1 gene. The results indicated that our established multiplex PCR is specific to detect the recurrent chromosomal translocations and able to identify common structurally genetic breakpoints of TEL-AML1 and E2A-PBX1 in ALL patients.

**Discussion**

Conventional cytogenetic, FISH, and molecular genetic testing play an important role for the diagnosis, risk stratification, planning of the effective therapeutic strategies, and disease monitoring in hematological malignancies. The detection of Philadelphia (Ph) chromosome by karyotyping is recognized as a genetic hallmark of CML (> 90 % of CML) and the test becomes a gold standard method for the first line diagnosis of CML (Tahira et al., 2015). In addition, approximately 5 % of ALL patients (childhood) harbor Ph chromosome which is recognized as a poor prognosis marker in ALL (Look, 1997). At the present, Imatinib which inhibits the tyrosine kinase activity of BCR-ABL fusion protein becomes an effective standard therapy for CML. The drug is able to induce complete hematologic response as well as complete cytogenetic response (CCyR) in a majority of patients and approximately 80 % of patients show complete remission. Thus, molecular genetic testing such as PCR-based technology is necessary for the monitoring of minimal residual disease in CML who displays the disappearance of Ph chromosome (CCyR). In this report, we were able to develop a highly sensitive multiplex PCR to detect BCR-ABL fusion transcripts including two variants of BCR-ABL.
However, in Iraqi pediatric ALL patients, TEL-AML1 and resulting in the formation of TEL-AML1 was recognized in ALL (age 2-11; median age = 4 for TEL-AML1 and 6 for E2A-PBX1) which was accounted for 8.41 % (18/241) cases of ALL. For other fusion transcripts, we could identify at least 10 fusion variants of MLL-AF4 that are able to be confirmed and investigated as poor prognosis markers in acute leukemia (van Dongen et al., 1999; Yang et al., 2010). Nevertheless, in this work we were not able to detect cryptic translocation of t(4;11) (q21;q23) (MLL-AF4) in the tested samples. MLL-AF4 fusion transcripts and other MLL rearrangements are recognized as poor prognosis markers in acute leukemia (Krivtsov and Armstrong, 2007; Muntean and Hess, 2012). In conclusion, we proposed that our generated multiplex PCR for routine use is a specific, highly sensitive, and fast method for screening and monitoring of recurrent chromosomal translocations in CML and ALL patients. This assay will provide an efficient tool for the clinicians to evaluate genetic status of the patients prior to clinical management decision.

Acknowledgements

We thank Dr. Suporn Chuncharuean and staffs at Division of Hematology, Department of Medicine, Faculty of Medicine Ramathibodi Hospital, Mahidol University for their valuable help especially for the ALL samples and clinical data. The authors would like to thank Ramathibodi Cancer Center for the support of reagents and chemicals.
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