Short Communication

Chimerism Analysis of a Patient with Acute Lymphoblastic Leukemia after Allogeneic Haematopoietic Stem Cell Transplantation: A Preliminary Report

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Abstract
A chimerism analysis for monitoring donor DNA engraftment in allogeneic peripheral blood stem cell transplantation (PBSCT) in a patient with acute lymphoblastic leukaemia (ALL) at day 14 (D14) and day 28 (D28) of post-PBSCT was performed. Five specific sequence polymorphisms located in five different chromosomes were selected for this study. Only one marker (S02) was able to discriminate DNA profiles between the donor and the recipient. This particular marker was negative in the recipient but positive in the donor. By using 15 serial-halved dilutions of mixed DNAs using TaqMan technology, a linear correlation ($r^2$) of 0.97 was obtained. In the post-PBSCT analysis, we managed to detect presence of the donor DNA profile at D14, which was slightly reduced at D28. The Ct values were 27.01 at D14 and 29.38 at D28. The percentage of donor DNA detected in the recipient’s DNA decreased from 37.18% at D14 to 30.68% at D28.

Keywords: Chimerism, Real-Time PCR, peripheral blood stem cell transplantation (PBSCT), acute lymphoblastic leukemia (ALL)

Introduction
Analysis of donor chimerism has become a routine method for documentation of engraftment after allogeneic haematopoietic stem cell transplantation (HSCT). The major causes of treatment failure are disease relapse, graft rejection and graft-versus-host disease (GVHD) phenomenon. Chimerism analysis has become an important tool for pre-transplant surveillance of engraftment. It offers possibility to realize impending graft rejection and can serve as an indicator for recurrence of underlying malignant or nonmalignant disease. More recently, chimerism analysis has become a basis for treatment intervention, such as to avoid graft rejection, to maintain engraftment or to treat imminent relapse by preemptive immunotherapy [1, 2]. The quantitative analysis of donor cells in patients with post-allogeneic stem cell transplantation is an important diagnostic tool for monitoring engraftment, early detection and treatment of graft rejection and disease relapse [3-6].

Haematopoietic chimerism can be assessed based on differences of polymorphic genetic markers between the donor and the recipient [5, 7-9]. A new quantitative polymerase chain reaction (Q-PCR) method using TaqMan technology has been established to detect single nucleotide polymorphism (SNP). SNPs are biallelic variants that differ at a single DNA base pair and they are widespread in human chromosomes within both coding and non-coding regions. It is estimated that SNPs occur at an average frequency of 1/1000 bp [4]. Chimerism status runs parallel with disease evolution and prognosis. The measurement of donor chimerism after transplantation is prerequisite for manipulating engraftment by altering patient immunosuppression and donor lymphocyte infusion [10].

In this study, we report the detection and quantification of a donor DNA in an allogeneic peripheral blood stem cell transplantation (PBSCT) patient with acute lymphoblastic leukaemia (ALL) at day 14 (D14) and day 28 (D28) of post-PBSCT using TaqMan technology and SNP-based analysis. This preliminary finding could open a new platform for a routine technique in assessing and monitoring patients with stem cell transplantation.

Materials and Methods

Patient and donor
The study involved a 4-year-old female ALL patient who underwent PBSCT. Based on the cytogenetic report, this patient had multiple chromosome abnormality, which includes trisomy 4, trisomy 14, trisomy X, deletion 5p, deletion 6q, deletion 21q (X2) and 2 Philadelphia (Ph)

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Chromosomes. PBSCT was received from an HLA-identical 11-year old male donor.

Before transplantation, fluorescent in situ hybridization (FISH) was carried out to confirm presence of translocation in the patient. Three positive fusion signals for BCR/ABL were observed in 236 (69.8%) out of 338 interphase nuclei, which indicated the presence of 2 Ph chromosomes (data not shown). Reverse transcriptase-polymerase chain reaction (RT-PCR) assay using Hemavision-7 System was also carried out for the simultaneous detection of seven most common leukemia translocation/inversion, including the most commonly occurring variants thereof namely, t(1;19), t(12;21), inv(16), t(8;21), t(4;11), t(15;17) and t(9;22). RNA from the K562 cell-line was used as a positive control for t(9;22)(q34;q11) BCR/ABL p210. Positive results were obtained for t(9;22) (q34;q11) BCR/ABL p190 and t(1;19).

Sample preparation

Pre-transplant (D0) peripheral blood samples from the patient and donor, and post-transplant (D14 and D28) bone marrow samples from the recipient were collected for chimerism studies. DNA was extracted from the peripheral blood and bone marrow nuclear cells using a QIAamp DNA Blood Midi Kit (QIAGEN, Germany) following standard protocol provided. The concentration and purity of DNA were measured by optical density at 260 and 280 nm with a Biospectrophotometer (Eppendorf).

Selection of genetic markers and primers and design of probes

Nucleotide sequences of polymorphic genetic markers selected from human biallelic short insertion/deletion polymorphisms were obtained from the Marshfield Clinic (Marshfield, WI, USA) (http://research.marshfieldclinic.org/genetics/GeneticResearch/DIDP.asp). The criteria used for selecting informative markers include biallelic polymorphisms, differing by at least two consecutive variable bases and showing a high level of heterozygosity in the general population. The markers, primers and probes used in the analysis were of that previously published by Alizadeh et al. [4] and is listed in Table 1.

### Table 1: List of markers, primers and probes used (obtained from Alizadeh et al. [4]).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Location</th>
<th>Informativity % Position</th>
<th>5' Primer 3</th>
<th>TaqMan (3' FAM-TAMRA 5')</th>
</tr>
</thead>
<tbody>
<tr>
<td>S01</td>
<td>17q</td>
<td>23.6</td>
<td>F GGTACCGGGTGTCACATGA</td>
<td>CTGGGCCAGAATCTTGGTCCTCACA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R GGGAAAGTCACCTACCAAGG</td>
<td></td>
</tr>
<tr>
<td>S02</td>
<td>Y</td>
<td>34.6</td>
<td>F GCTCTCTGGTTGGAGTCACG</td>
<td>GCAGCACACACACCCGATGTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R GCTTGCTGGCGGACCCT</td>
<td></td>
</tr>
<tr>
<td>S05a</td>
<td>20</td>
<td>1.8</td>
<td>F AGTTAAAGTAGACACGGCCTCCC</td>
<td>CAGCATCTAGCAGCTATTCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R TCTACCTTCTACCTACCT</td>
<td></td>
</tr>
<tr>
<td>S06a</td>
<td>X</td>
<td>31.0</td>
<td>F TGGTATTGGCTTTTATAATCTGGG</td>
<td>TGGGCAAGCTAGCAGCAGTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R TCTACCTTCTATCTACCTT</td>
<td></td>
</tr>
<tr>
<td>S09a</td>
<td>11</td>
<td>25.5</td>
<td>F TAGGATCCACCCCTGGAAGC</td>
<td>CAGGCTTCCTCAATTCTTCACCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R CAGCACTGCACCTGACAT</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td>F GGACTGAGGCTTCCACCTTT</td>
<td>CATTGCAGACTTG CCTCCTTCG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R GCAATGGACTGTGGTCTGAA</td>
<td></td>
</tr>
</tbody>
</table>

Real-time quantitative PCR

Real-time quantitative PCR was performed using TaqMan technology with dual-labeled fluorogenic hybridization probe (FAM-TAMRA) and ABI PRISM 7300 apparatus (Molecular Diagnostic and Protein Unit, Specialized Diagnostic Centre, IMR). The Ct value is defined as the cycle number at which a significant increase in the fluorescent signal that was first detected (the higher the starting copy number, the lower the Ct). PCR reaction mixture contained 204 ng DNA with 10 µl Master Mix Buffer (Quantitect Probe PCR Kit), 0.6 µM each primer, 0.2 µM TaqMan Probe in a final volume of 20 µl. PCR cycles profile was 2 min at 50°C, followed by 10 minutes at 95°C and 40 amplification cycles (95°C for 45 sec and 60°C for 60 sec).

Standard amplification curves

Standard amplification curves were plotted for recipient- and donor-specific alleles. DNA samples made with 15 serial halved dilutions of donor DNA in order to evaluate the validity and sensitivity of real time quantitative PCR chimerism analysis.

Real-time quantitative PCR chimerism assay

Before quantification, donor and recipient samples were screened for 5 biallelic loci (S01, S02, S05, S06 and S09) using the TaqMan technology. Positive alleles were defined by a Ct value ranging between 27 and 33, whereas alleles were negative if the Ct values exceed 36. An allele was considered to be informative or polymorphic when positive in the recipient DNA and negative in the...
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Donor DNA or negative in the recipient DNA and positive in the donor DNA. When more than one allele is informative, only one marker was used for each genotype profile. The chimerism assay protocol was performed on post-BMT DNA samples as well as pre-BMT recipient and donor DNA samples, recipient-specific allele amplification, donor specific allele amplification and active reference amplification. All assays were performed in duplicate.

Results and Discussion

Chimerism assessment after allo-HSCT is routinely performed to monitor the outcome of engraftment. It has become the basis for treatment intervention, particularly in reduced intensity conditioning of allogeneic transplants for changing immunosuppressive therapy as well as for the treatment of disease relapse. The current routine detection methods such as sex mismatch, microsatellite analysis and red cell phenotyping techniques have relatively low sensitivity, are expensive and limited in their applications.

Five markers used in this study were based on SNP analysis with deletion and insertion of the alleles in the sequence of primers. Among them, only the S02 marker was informative, which was negative in the recipient but positive in the donor. The rest of the markers were positive in both the donor and the recipient, whilst marker S09a failed to amplify in both the donor and the recipient. By using 15 serial-halved dilutions of mixed DNAs, the linearity of the method was evaluated and a linear correlation \( r^2 = 0.97 \) was obtained (Figure 1a). In the post-PBSCT analysis, we managed to detect presence of donor-DNA profile, which was higher at D14 and slightly reduced at D28. The Ct values for D14 and D28 were 27.01 and 29.38, respectively (Figure 1b). The percentage of the donor’s DNA detected in the recipient’s DNA decreased from 37.18% at D14 to 30.68% at D28.

Detection of the donor’s DNA in the recipient clearly shows that the stem cells from donor’s peripheral blood were successfully transplanted to the patient. By using real time PCR, success of this transplantation could be interpreted by the percentage of donor’s DNA in the recipient DNA, which was 37.18%. This amount was calculated by mean of donor DNA and active reference (GAPDH). This is supported by cytogenetic studies at post-PBSCT, where male karyotype of the donor was observed in the recipient’s cells (data not shown). Chimerism evaluation after allo-BMT is crucial in many situations. Studies have shown that chimerism status closely correlates with disease evolution and could be used for prognosis indication [11, 12].

Real-time Q-PCR technique provides the ability to analyze more than one marker in the same run and is therefore a feasible diagnostic tool for chimerism studies. On the other hand, the real time quantitative PCR procedure measures the quantity of PCR product at the onset of the exponential phase, which is directly proportional to the initial amount of the target DNA sequence. Several methods have been extensively used for assessment of chimerism status in allogeneic transplantation and gives different sensitivity in detection of MRD after treatment. FISH detection of chromosomal translocation has a level of sensitivity between 0.1% and 8% depending on the technique used and the specific translocation investigated [13]. However, this technique is of poor informativity and restricted to the sex-mismatched allografts. Flow-cytometry assays monitoring aberrant antigen expression, might achieve considerably higher levels of sensitivity of between 0.01% and 1% [14].

Advancement in the PCR technique has revolutionized the approach of chimerism analysis. The exponential amplification of specific target sequences facilitates a limit of detection between 0.1 and 0.001% cells [15]. Thus, fluorescent-based PCR analysis of short tandem

Figure 1: Amplification standard curve generated from DNA-allele-specific with 15 serial-halved dilutions of recipient/donor DNA. Artificial chimeric DNA samples were made with serial-halved dilutions of donor DNA in recipient DNA (from 100% to 0.01%). Panel (a) shows a standard amplification curve plotted from recipient marker Ct values, which correlated linearly with the logarithm of recipient/donor DNA fraction \( r = 0.977 \). Panel (b) shows a quantification curve from TaqMan probe-based Q-PCR. The X-axis indicates the cycle’s number when PCR started generating fluorescent signals.
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Chimerism analysis of an ALL patient after allogeneic HSCT has become the gold standard for quantitative chimerism analysis so far. This method offers the highest informativity (nearly 100% of unrelated allografts are evaluable) and good accuracy for quantification [3]. However, this technique is relatively expensive and the sensitivity of this method, which is between 0.4% and 5%, is low [4].

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References
