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Original Research Article

Comparison of Quantitative Real-Time PCR and Short Tandem Repeat PCR for Monitoring Chimerism after Hematopoietic Stem Cell Transplantation in Paediatric Patients

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ABSTRACT

Post-hematopoietic stem cell transplantation chimerism monitoring is important to assess the kinetics of engraftment, and to assist in diagnosis of graft failure, rejection or relapse. The purpose of this study aimed to compare two methods, short tandem repeat polymerase chain reaction (STR-PCR) and quantitative real-time PCR (qRT-PCR), in order to determine hematopoietic chimerism. The chimerism of blood samples from 57 paediatric patients were analyzed by STR-PCR and qRT-PCR on different days after allogeneic hematopoietics stem cell transplantation. The results showed good quantitative correlation between the two methods with correlation coefficient (r)=0.854 (p<0.001). The high correlation between STR-PCR and qRT-PCR methods helps to validate the use of qRT-PCR for chimerism analysis. The chimerism status may differ using the different methods. In some samples, the chimerism status using the STR-PCR showed complete donor chimerism however it showed mixed chimerism using qRT-PCR. On the other hand, all samples with complete donor chimerism in qRT-PCR were also in complete donor chimerism using STR-PCR. Thus, qRT-PCR is a valid, more sensitive, simple and rapid compared to STR-PCR method in chimerism monitoring.

Key words: chimerism, qRT-PCR, STR-PCR, transplantation, sensitivity

INTRODUCTION

The assessment of donor-recipient chimerism is an important component in routine monitoring of patients after haematopoietic stem cell transplantation (HSCT) in order to predict the success or failure of the treatment. Continuous complete engraftment of donor derived hematopoiesis is considered essential for the success of the treatment as it can prevent reappearance of underlying disease. [(1)] Treatment failure is usually due to the three major causes, namely, disease recurrence, graft rejection and graft-versus-host disease (GVHD). [(2,3)] A quantitative method to assess chimerism status is therefore important to accurately determine the proportion of donor cells engraftment in post-transplant recipients as well as to

predict impending rejection and disease recurrence. [4]

Following HSCT, a dynamic relationship exists between engrafted cells and recipient which is often reflected as fluctuating chimerism. [5] It is known that donor and recipient hematopoiesis could co-exist after HSCT in the recipient. The state of co-existence of hematopoietic cells is called mixed chimerism. However, if all hematopoietic cells post-transplant are of donor origin, the recipient is called a complete chimera and shows a complete chimerism. As the evolution of post-transplant chimerism is dynamic, many recipients with complete donor chimerism can later develop a state of mixed chimerism and vice versa at a certain time point after transplant. [6] On the other hand, the degree of recipient cells in recipients with mixed chimerism state can also be increased or decreased. These recipients are then considered to have an increasing or decreasing mixed chimerism.

Peripheral blood or bone marrow aspirate is most often used for chimerism analysis. [7] Several techniques have been applied for the test during the last 30 years. [8] Polymerase chain reaction (PCR) based procedures have been developed for the evaluation of engraftment. Most of these assays rely on the amplification of highly polymorphic repetitive DNA sequences such as short tandem repeat (STR). The fluorescent based PCR analysis of STR is the gold standard for quantitative chimerism analysis. Quantitative real time-PCR (qRT-PCR) using TaqMan technology is another method for determining chimerism and it relies on the detection and measurement of the PCR process itself. [2] The sensitivity of the applied method has an impact on the degree of chimerism. A patient could be reported as a complete chimera when the chimerism testing was carried out using a less sensitive technique, but as a mixed chimera when the test was carried out by a more sensitive technique. The aim of this study is to find a more accurate and efficient methodology for surveillance of chimerism in recipients after HSCT using two methods: qRT-PCR vs. STR-PCR.

MATERIALS AND METHODS

Patients

This study was conducted among paediatric patients after getting approval from the Medical Research & Ethics Committee (MREC), Ministry of Health, Malaysia. There were 57 children with various diagnoses, which included severe aplastic anemia, thalassaemia, leukemia and others. All of them had undergone allogeneic HSCT from HLA-matched siblings in Paediatric Institute, Hospital Kuala Lumpur (HKL) from year 2011 to 2012.

Chimerism Analysis

The analysis was performed using two different methods: qRT-PCR and STR-PCR. We used peripheral blood samples that were collected from recipients and their respective donors prior to transplantation and from recipients at different times point after transplantation for the analysis. A total of 147 blood samples from 57 patients were collected.

DNA Extraction

Genomic DNA was extracted from peripheral blood using a QIAamp DNA blood midi kit (QIAGEN, Germany) according to manufacturer's instructions. The concentration and purity of each DNA sample was evaluated by means of measurement of optical density at 260 nm and 280 nm with a spectrophotometer (Nanodrop, USA).

Quantitative Real-time PCR

There were three major steps in the qRT-PCR technique: (a) genotyping, (b) plotting standard amplification curve and (c)

quantification of chimerism. In this study, all steps were performed by means of TaqMan technology using the LightCycler 480 apparatus (ROCHE, Germany). The sequences for the primers and probes for the 19 selective loci and the GAPDH gene were published previously. [2] The PCR reaction parameters were as follows: (I) reaction mix: 250 ng DNA with LC480 Probe Master (ROCHE, Germany), 600 nM each primer, and 200 nM TaqMan probe in a final volume of 20 µl; (II) PCR cycles: 2 min at 50 °C followed by 10 min at 95 °C and 40 amplification cycles (95 °C for 45 s and 60 °C for 60 s).

Donor and recipient genotype were initially identified before HSCT to find the informative recipient marker. 125 ng DNA was added in each well, and qRT-PCR was carried out. A genetic marker was considered informative for recipient when it was positive on recipient DNA and negative on donor DNA. When more than one informative genetic marker observed in recipient, only one marker gene was chosen for chimerism analysis since quantitative analyses were similar regardless of genetic marker type. [2]

After genotyping, a standard curve was plotted for the selected informative recipient-specific allele and reference gene. We used the plot to evaluate the validity, sensitivity and efficiency of the technique; and also as a reference to determine the concentration of recipient DNA in post-transplant sample. For this purpose, 14 serial halved dilutions were prepared from the artificial chimeric DNA that made from the mixture of pre-transplant recipient and donor DNA. The DNA ratio were ranging from 100% to 0.01% with a constant final amount of 250 ng chimeric DNA. The amplification curve was plotted for each tube using LightCycler software by relating the fluorescence signal intensity to the cycle number. The cycle number on the first detection of significant increased in the fluorescence signal was referred as a crossing point (Cp) value. Standard curve was generated on the basis of the relationship between Cp value and the logarithm of the recipient DNA copy number.

Finally, quantification of chimerism was performed to assess chimeric status after HSCT. In this step, the Cp values of the selected genetic marker and reference gene (GAPDH) were first acquired for pre-HSCT and post-HSCT recipient sample. Next, the DNA concentration of the specific marker and reference gene were determined by referring the obtained Cp values to the respective standard amplification curves that have been plotted. Then the normalized values of the specific marker gene for both samples were calculated by LightCycler software based on the use of individual PCR efficiencies of target and reference gene. In this method, the pre-HSCT recipient sample was indicated as a calibrator and the normalized value of the calibrator was considered containing 100% fraction of recipient DNA. Based on this principle, the percentage of recipient DNA was determined by calculating the percentage for the ratio of normalized value of post-transplant sample to normalized value of calibrator. [9] The percentage of donor DNA was then derived by subtracting the percentage of recipient DNA from 100%.

STR Amplification

Conventional PCR was employed to discover the length of repeated nucleotides in STR loci. We used AmpFISTR Identifiler PCR amplification kit (Applied Biosystems, USA). The primers used in this method were locus specific containing 6-FAM, VIC, NED and PET dye-labelled and unlabeled. The final volume was 12.5 ul that included 0.625 ng of DNA per reaction. The thermal cycling conditions were: 95 °C for 11 min, followed by 28 cycles of 94 °C

for 1 min (denature), 59 °C for 1 min (annealing) and 72 °C for 1 min (extension). The final extension step was 60 °C for 60 min. The amplified PCR products were then separated and detected by capillary electrophoresis using an ABI3730xL DNA analyzer (Applied Biosystems, USA) before being analyzed by GeneMapper ID v3.2 software.

Prior to transplantation, both donor and recipient samples were analyzed to identify the informative STR region. Similar like qRT-PCR, these informative markers were used for chimerism testing in post-transplant recipient sample. The results were then analysed using GeneMapper ID v3.2 software to estimate the length of the informative STR loci for both donor and recipient. From here, the percentage of donor loci were calculated based on the formula suggested by Nollet *et al.* [(10)]

Statistical Analysis

All statistical analyses were conducted using SPSS software version 18 (IBM SPSS, Chicago). Scatter plot was used to demonstrate the percentage of donor DNA by STR-PCR plotted against the percentage of donor DNA for qRT-PCR. The best fit line was drawn by linear regression analysis. The coefficient of determination, denoted R^2, represents how well data points fit the line. The more data points cluster closely around the best fit line, the stronger the relationship that exists between the two methods. The R^2 can range from 0 to 1. The $R^2 = 1$ when regression line passes exactly through every point on the scatter plot. Pearson's correlation test was conducted to examine the linear relationship between the STR-PCR and qRT-PCR results. The Pearson's correlation coefficient (r) was used to measure the strength and direction of association between the STR-PCR and qRT-PCR methods. The value of r can vary from -1 to +1. A -1 indicates a perfect negative correlation, while a +1 indicates a perfect positive correlation. A correlation of zero means there is no relationship between the two methods. The value of $r > 0.8$ indicates that there is a good correlation between the two methods. For all analyses, p values of less than 0.05 were considered statistically significant.

RESULTS

Demographics of Patients

A total of 57 paediatric patients who received HSCT were studied. The ages of patients ranged from one to 18 years and the median age was seven years. The main races were Malays, Peribumi Sabahans and Chinese, followed by Indians, Peribumi Sarawakians and others. These patients were mostly diagnosed with acute lymphoblastic leukemia (ALL), beta thalassemia major, acute myeloid leukemia (AML), severe aplastic anaemia and others. The details of the patients' characteristics are shown in Table 1.

Comparison Between qRT-PCR and STR-PCR Chimerism Analysis

For the analysis of post-transplant chimerism, only 44 out of 57 patients were evaluable. A total of 109 samples from these 44 patients taken at different days post-transplant were compared by using qRT-PCR and STR-PCR methods. Thirteen patients were excluded due to absence of informative markers, insufficient pre-transplant DNA of recipient and unacceptable PCR efficiency in the test run. In our study, the informative gene markers detected by qRT-PCR were only 87% of recipient-donor pairs, lower than seen in STR-PCR (98%).

The correlation curve (Figure 1) obtained from the quantification of chimerism with qRT-PCR and STR-PCR illustrates that the percentage of donor DNA in qRT-PCR results is slightly higher than the corresponding STR-PCR results. Slight

overestimation of the percentage of donor DNA by qRT-PCR analysis or slight underestimation by STR-PCR analysis may account for the difference in the qRT-PCR and STR-PCR results. The overall correlation of qRT-PCR and STR-PCR results is good with an R^2 value equal to 0.729 and a significant p value ($p < 0.001$).

Table 1. Characteristics of patients.

Number of patients	57
Mean age in years (range)	7 (1-18)
Sex, n (%)	
Male	29(51)
Female	28(49)
Race, n (%)	
Chinese	7(12)
Indian	4(7)
Malay	31(54)
Peribumi Sabah	10(18)
Peribumi Sarawak	2(4)
Others	3(5)
Diagnosis, n (%)	
ALL	13(23)
Beta Thalassemia Major	13(23)
AML	8(14)
Aplastic Anaemia	8(14)
CML	5(9)
MDS	3(5)
Infantile Leukemia	2(4)
Pure Red Cell Aplasia	2(4)
Alpha Thalassemia Hb H	1(2)
Chronic Granulomatous Disease	1(2)
JMML	1(2)
Recipient/Donor sex, n (%)	
Male/Male	19(33)
Male/Female	10(18)
Female/Male	10(18)
Female/Female	18(32)

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; MDS, myelodyplastic syndromes; JMML, juvenile myelomonocytic leukemia

Table 2. Chimerism status obtained from qRT-PCR and STR-PCR methods.
MC: Mixed chimerism CC: Complete donor chimerism

Chimerism Status		STR-PCR		Total
		MC, n(%)	CC, n(%)	
qRT-PCR	MC, n(%)	83 (76.2)	24 (22.0)	107 (98.2)
	CC, n(%)	0 (0%)	2 (1.8)	2 (1.8)
Total		83 (76.2)	26 (23.8)	109 (100)

A total of 109 samples was analyzed by qRT-PCR and STR-PCR to compare their effectiveness in chimerism monitoring (Table 2). Our study showed that there was 78% agreement of chimerism status (2 samples were identified as CC and 83 samples with MC) identified by both methods. However, 24 samples identified as

MC by qRT-PCR were defined as CC by STR-PCR. This showed higher sensitivity of the qRT-PCR technique.

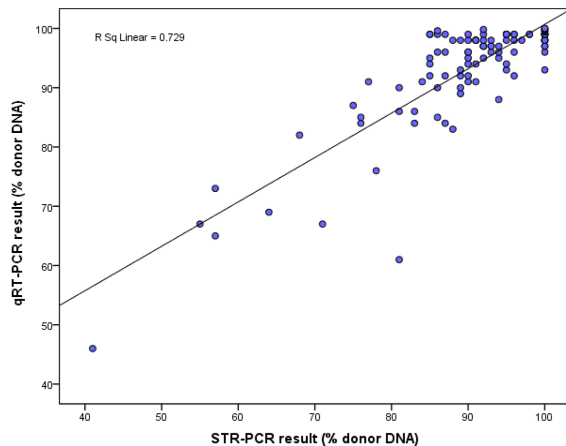

Figure 1. Comparison of chimerism quantification results between STR-PCR and qRT-PCR. Linear regression analysis shows good correlation of STR-PCR and qRT-PCR analyses for patient post-transplant samples.

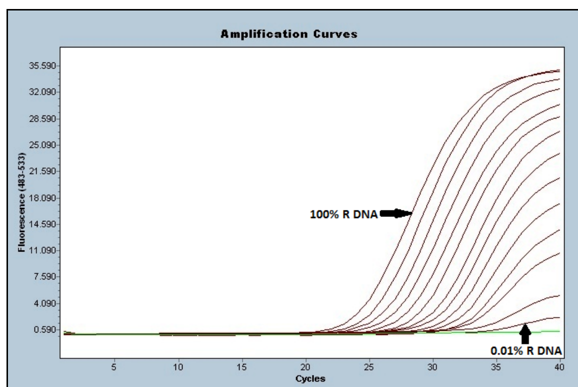

Figure 2. Amplification curve for the 14 chimeric samples.

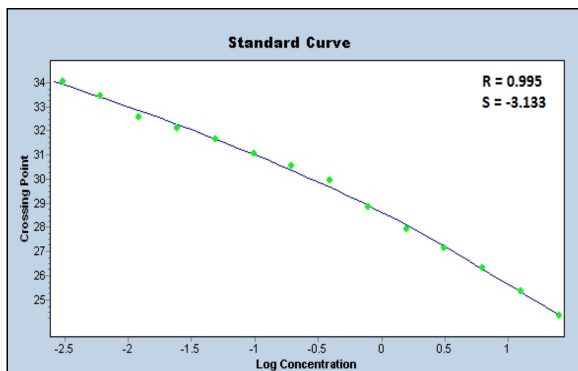

Figure 3. Standard curve plotted from the recipient marker Cp values, versus logarithm of recipient/donor DNA fraction (r = 0.995).

Sensitivity

In STR-PCR, sensitivity to the minor component DNA was not examined because it had been evaluated in a number of laboratories and is in the range of 1-5%. [5,11]

The standard amplification curve was performed by qRT-PCR and shown in Figure 2. The amplification plots were shifted to the right to higher threshold cycles where Cp values increased approximately by 1 when the input of recipient DNA quantity was reduced in the dilution. A linear regression analysis was performed with an r value of 0.995 and negative slope illustrating that qRT-PCR technique is highly sensitive (Figure 3). This illustrates that qRT-PCR method has a high linear correlation between logarithm of recipient/donor DNA concentration and recipient marker Cp values; thus it has the ability to detect a minimum of less than 0.1% cells of interest.

DISCUSSION

Quantitative monitoring of chimerism after hematopoietic stem cell transplantation can predict engraftment, graft rejection or relapse. [12,13] In fact, prediction of relapse has been found at chimerism levels ranging from 0.1 to 10%, depending on the post-transplantation time and the individual kinetics. [14] An appropriate quantitative method is hence needed for the follow-up of transplanted patients.

Presently, STR-PCR is the gold standard method for quantitative chimerism analysis. It offers the highest informativity for quantification. Informative results with STR-PCR could exceed 98% of recipient/donor pairs. However, the STR-PCR method is less precise as data are collected at the end point of the PCR reaction. Furthermore, the quantity of PCR products is evaluated, whose level depends on a large number of variables. Stutter peaks, which are artifacts and polymerase

slippages are other problems encountered which may interfere with engraftment analysis. [15] Amplification of the minor component of DNA in a standard PCR method such as STR-PCR requires a longer amplification time. The data analysis for STR-PCR is also more complex and time consuming.

On the other hand, qRT-PCR is based on TaqMan probe technology and gives a high dynamics of detection range. With respect to informativeness, more than 87% of the recipient/donor pairs in this study could be analyzed using the 19 markers published by Alizadeh *et al.* [2] qRT-PCR measures the quantity of PCR product at the onset of the exponential phase which is more precise and accurate. The amplification step is short in qRT-PCR because it specifically targets the minor component DNA only. qRT-PCR result analysis could be obtained in minutes.

In order to ensure early detection of the reappearance of recipient hematopoiesis and to enable the appropriate clinical decisions to be taken promptly, the highest sensitivity should be available for the follow-up of patients. To test the sensitivity, a serial dilution of recipient and donor is performed as discussed in Method. These artificial samples were used to test the quantification accuracy of the techniques as well as the reproducibility of the techniques. qRT-PCR has better sensitivity with detection limit of less than 0.1% depending on the amount of DNA used, compared to STR-PCR detection limit of 1-5% only. [16] The sensitivity of STR-PCR is relatively low, mainly as a consequence of PCR competition biases.

Results obtained from qRT-PCR for patient samples were also compared with STR-PCR results based on chimerism status as shown in Table 2. When chimerism status of qRT-PCR showed CC, it was also CC in STR-PCR. However when chimerism status of STR-PCR results showed CC (n=26), results in qRT-PCR was not necessarily CC. This is because STR-PCR cannot detect recipient DNA in the patient hematopoietic system, but qRT-PCR still can detect very minute amounts of recipient DNA in the same patient. This indicates higher sensitivity of the qRT-PCR method.

This study also showed a good correlation between qRT-PCR and STR-PCR analyses for the same sets of patient post-transplant samples with *r* value more than 0.8 ($p < 0.001$). Hence, qRT-PCR analysis is a reliable method for monitoring engraftment because it has good correlation with STR-PCR analysis which is the gold standard for chimerism analysis.

This study demonstrated that both qRT-PCR and STR-PCR methods are reliable tools and either method can be integrated into a laboratory for chimerism analysis to monitor engraftment. However, qRT-PCR has greater sensitivity and linearity over STR-PCR. [17] qRT-PCR can reduce hands-on time and thus improves turn around time. Non informative markers were among the problems encountered in qRT-PCR method. Although the informative results for qRT-PCR using these markers could reportedly exceed 90% (Alizadeh *et al.* [2]), in our study, the informativeness was only 87%. This could be due to the use of markers which were chosen for the European population. Selection of suitable markers specific to our population could perhaps improve informativeness for the qRT-PCR chimerism assay. In those cases with no informative markers, STR-PCR will be used for the chimerism analysis. The cost of commercial STR kits is relatively high and is hence more suitable to run large volume of samples.

CONCLUSION

In conclusion, we have compared qRT-PCR and STR-PCR methods for

quantification of chimerism and found that either method can be used as a method of choice. The decision whether to use qRT-PCR or STR-PCR in a molecular diagnostics laboratory would depend on the quantity of DNA available, time, cost, test volume, available instrumentation and laboratory personnel. Guidelines or standards for chimerism monitoring need to be established by laboratory professionals and clinicians for better therapeutic intervention and better patient care.

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