QUANTITATIVE EVALUATION OF CHIMERISM STATUS FOLLOWING HAEMATOPOEITIC STEM CELL TRANSPLANTATION USING A MICROCHIP ELECTROPHORESIS SYSTEM

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ABSTRACT: We aimed to establish a method for quantitative analysis of mixed haematopoietic chimerism based on microchip electrophoresis of selected molecular markers following PCR amplification for accurate monitoring of graft status post-transplantation. A 12-year-old girl with relapsed acute lymphoblastic leukaemia who underwent allogeneic bone marrow transplantation had qualitative chimerism analysis using short tandem repeat markers at three time points following the procedure. Her archived DNA samples were then used to test the ability to correlate her clinical course with changes in the quantity of donor chimerism at the different time points. Quantitative chimerism analysis was performed on the Agilent 2100 bioanalyser and donor-recipient ratios were calculated from generated electropherograms. Complete donor chimerism (98%) was demonstrated three weeks post- transplantation. Decreasing amount of donor chimerism to 24% was shown after three months and this concurred with clinical relapse. Following a second transplant, full donor chimerism was re-established where donor chimerism rose to 100%. High resolution microchip electrophoresis could be useful in predicting the occurrence of increasing recipient chimerism which may herald impending relapse in patients while the disease burden is still low. This investigational approach may provide useful information for clinicians to select appropriate intervention strategies to ensure successful transplantation. *(JUMMEC 2007; 10(1): 11-16)*

KEYWORDS: Haemopoeitic stem cell transplantation (HSCT), chimerism analysis, Short Tandem Repeats (STR)

Introduction

Haemopoeitic stem cell transplantation (HSCT) has been used in the treatment of many malignant and non-malignant disorders in children since the 1970s. The paediatric HSCT unit of the University of Malaya Medical Centre (UMMC) was established in 1987 and has performed more than 300 transplants from both sibling and unrelated cord blood donors.

Following transplantation, it is of great importance to determine whether engraftment has occurred successfully. The investigation of the genotypic origin of post-transplant haematopoeisis is called chimerism analysis. Since chimerism analyses were first performed, many different methods have been developed. The basic principle in these studies has been demonstrating differences (or similarities) of genetic markers between the donor and recipient (1). Early methods included red cell phenotyping (2) and determination of sex chromosomes by either conventional karyotyping or fluorescence in situ hybridization (FISH) (3). However, these methods are not feasible in patient-donor pairs who share red cell antigen types or those who are of the same gender.

In the 1990s, detection of molecular markers, namely, variable number of tandem repeats (VNTR) or short tandem repeats (STR) was developed for chimerism studies (4,5). The paediatric oncology research laboratory of the UMMC commenced chimerism

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studies using PCR amplification of STR markers in April 2005. To date, engraftment information has been provided for over 30 adult and paediatric patients. However, clinical decisions for the management of mixed chimerism state have been hampered by the lack of quantitative data and the inability to monitor trends in donor-recipient ratios.

To overcome this problem, we developed a method to accurately quantify donor chimerism where this information can aid clinical decisions with regards to manipulating a graft, for example using donor lymphocyte infusion. Here, we demonstrate the use of microchip electrophoresis for quantitative chimerism analysis and correlate it with a patient's clinical course.

Patient and Methods

Case Report

MW is a 12-year-old girl with relapsed acute lymphoblastic leukaemia, who underwent allogeneic bone marrow transplantation at the Subang Jaya Medical Centre (SJMC), Selangor in November 2005. Her elder sister was her donor. Marrow samples were obtained at three time points (TP1, TP2 and TP3) which were at week 3 and at the third and fourth months, respectively, after her transplantation. Amplification of short tandem repeats (STR) DNA followed by PAGE electrophoresis were used to assess donor chimerism.

Two STR markers (CSF1P0 and TH01) were found to be informative and able to provide information on engraftment status. Full engraftment was shown at week 3 post-transplant (TP1) depicted by sole presence of donor bands (Figure 1).

At three months of post-BMT (TP2), she was noted to have a rapid rise in the total white cell count. Chimerism studies done at this point in time showed the presence of a hitherto undetectable recipient band (Figure 2). Subsequent bone marrow examination revealed frank relapse of leukaemia. Since no sample was sent for STR monitoring between TP1 and TP2, we were not able to show the point where early prediction of relapse was possible.

Following chemotherapy, she underwent a second transplantation from the same donor in March 2006. Analysis done one month after the second transplantation (TP3) showed that she had changed to full donor chimerism. This was concordant with the presence of complete remission status by bone marrow morphology and by minimal residual disease analysis performed in Singapore. At the time of writing (8 months following the second BMT), she remains in continued clinical remission.

Genomic DNA extraction

Peripheral blood samples were obtained prior to BMT from MW and her donor and subsequently at three time points as stated above. DNA was extracted using the modified phenol chloroform method and ethanol precipitation (6). The extracted DNA was suspended in sterile nuclease free double distilled water and kept at 4°C.

Purity of DNA samples were checked and quantified prior to subsequent testing using an automated spectrophotometer (Eppendorf AG, Germany). Purity and concentration were estimated from the optical density ratio (OD260/OD280) with a range of purity between 1.7 and 1.9.

Multiplex STR Polymerase Chain Reaction

Multiplex STR amplification was performed using GenePrint™ CTT Multiplex System (Promega, Madison, WI, USA). Each kit simultaneously amplifies three autosomal tetranucleotide STR loci with non-overlapping allele size ranges. Loci in the CTT kit are CSF1PO, TPOX and TH01. PCR amplification was performed using 100 ng template DNA on a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). PCR was carried out in a final volume of 25 µl using 10X STR buffer, 10X multiplex primer pair mix (Promega) and 0.75 U of HotStar Taq™ DNA polymerase (Qiagen, Hilden, Germany). Ten nanograms of K562 DNA served as positive amplification control to validate the result.

Initial PCR consisted of activation of the polymerase at 96°C for 2 minutes, followed by the first 10 cycles of PCR amplification (denaturation at 94°C for 1 minute, annealing at 64°C for 1 minute and elongation at 70°C for 1.5 minutes). It was then followed by different PCR parameters for another 20 cycles where denaturation occurred at 90°C for 1 minute, annealing for 1 minute and elongation at 70°C for 1.5 minutes.

Quantitative PCR analysis

Archived PCR products taken at the three time points above were used in this study. Quantification of PCR products were accomplished using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, USA) in conjunction with the DNA 1000 Labchip kit. The chip contains 16 wells: 3 for loading of the gel fluorescent dye mixture, 1 for the molecular size ladder, and 12 wells for samples. One microlitre of each PCR product was used for analysis. The migration and automated analysis were completed in 30 minutes. Results were

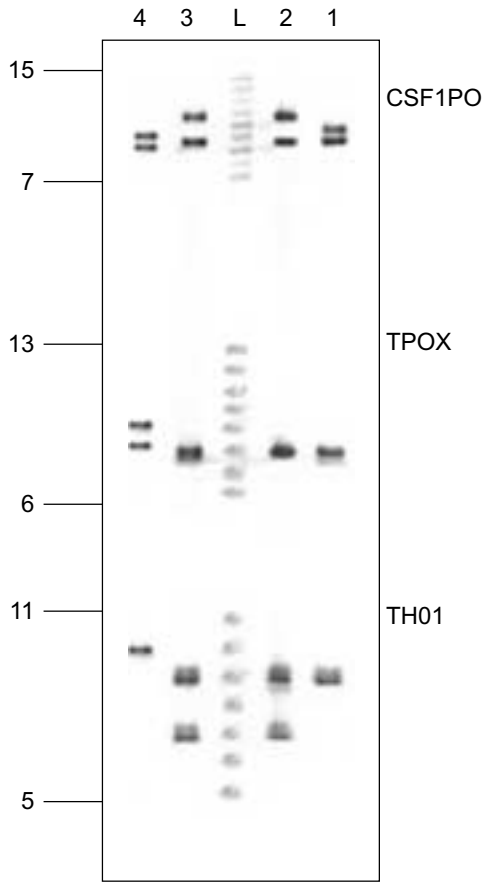


Figure 1. Full engraftment showed by STR PCR of CTT Multiplex System

Lane 1 : DNA from patient before transplant.
Lane 2 : DNA from donor.
Lane 3 : Donor specific alleles of two informative loci; CSF1PO and TH01 in week 3 post-transplantion sample.
Lane 4: K562 control DNA showing expected allele sized.
L : Allelic ladder showing number of repeat units form the respective STR loci.

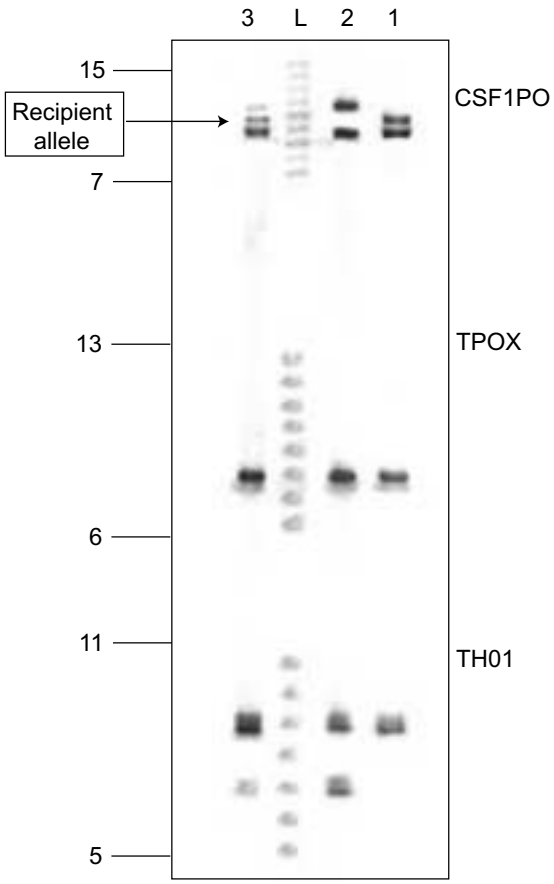


Figure 2. Mixed chimerism of donor and patient DNA, detected with STR PCR of CTT Multiplex System

Lane 1 : DNA from patient before transplant.
Lane 2 : DNA from donor.
Lane 3 : Donor specific allele 11 of CSF1PO locus was present together with donor specific allele; 3 months post-transplantation. There was a decrease in the relative intensity of the donor specific bands.
L : Allelic ladder.

shown in three formats: gel-like image, electropherograms and data table. The bioanalyzer software package was used to correlate allele peak areas to the percentage of donor or recipient DNA.

Results

Results of the quantitative chimerism studies are summarised in Figure 3. A sudden rise in the patient's peripheral white cell count, associated with disease relapse, was shown by decreasing amounts of donor chimerism to 24%. Following a second bmt (TP3), full donor chimerism was re-established where donor chimerism rose to 100%.

Discussion

One rationale for monitoring chimerism following allogeneic transplantation is that it may enable prompt therapeutic intervention to treat early relapse or rejection; leading to improved outcome. Also, analysis of chimerism may be of value as an alternative to monitoring minimal residual disease. The significance of mixed chimerism will depend on the time at which it is detected and the pattern of change in the proportion of recipient cells.

Quantification depends on identifying and measuring genetic markers that differ between the donor and the graft recipient. Full engraftment or complete chimerism refers to complete replacement of host cells by donor haematopoiesis. Also, no evidence of recipient cells should be present at any time post-transplantation (7). Quantitatively, it is defined by the presence of <2% of recipient cells in bone marrow or peripheral blood (8). This is the ideal haematological status and the patient is usually associated to be free of disease.

Mixed haematopoietic chimerism can be defined as the detection of 2.5-97.5% cells of donor origin in haematopoietic tissues, which approximately defines the sensitivity of routinely used assays for quantifying chimerism (5,9). In malignant diseases, persistent mixed chimerism state may be caused either by regeneration of normal recipient haematopoeitic cells (10) or emergence of leukaemic cells. Barrios *et al* reported in 133 leukaemia patients, those with increasing mixed chimerism of more than 5% of recipient cells have a significantly higher risk (94%) of developing relapse and death compared to patients who showed low levels of persisting recipient cells (11). Similar findings have been reported by Bertheas (12). The time interval between the detection of mixed chimerism and the onset of relapse was reported to range between five to 434 days (11, 13).

It is clear that it is not a mixed chimerism state per se but an increasing amount of recipient cells (or decreasing donor chimerism) which is important for prediction of relapse. Thus, serial quantitative analysis showing increasing recipient chimerism may identify a cohort of patients with impending relapse which may be prevented by pre-emptive immunotherapy.

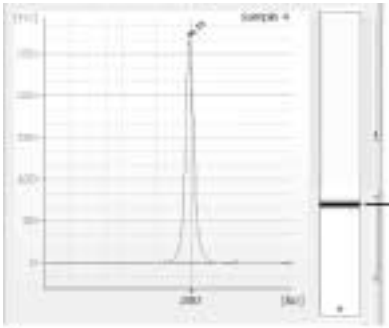
In the present study, analyses of recipient and donor DNA polymorphisms were carried out using the Agilent 2100 system that is capable of rapidly analysing small DNA fragments of up to 12 samples per run. A virtual gel image is also available together with electropherograms showing the fluorescent unit of each peak upon completion of electrophoresis.

In this series of follow-up samples, the donor and recipient alleles of TH01 were successfully separated since the base-pair size difference was more than 6 base pairs. The other informative STR locus CSF1PO was not analysed as the close base pair size difference of the recipient and donor alleles caused overlapping of the electropherograms. The DNA 1000 Labchip assay that we used is able to separate alleles with a size range of 25-1000 base pairs (14).

The time interval between HSCT and molecular analysis by STR or VNTR has been reported to affect the degree of mixed chimerism and prediction of relapse. The sensitivity of methods may be partly attributed to the degree of mixed chimerism detected. Mixed chimerism detection using STR has a sensitivity limit in the range of 0.5 to 2.5% to detect a band or peak for a minor cell population (7,15). Obviously, the usage of a more sensitive detection method will improve our ability to detect the occurrence of mixed chimerism in future.

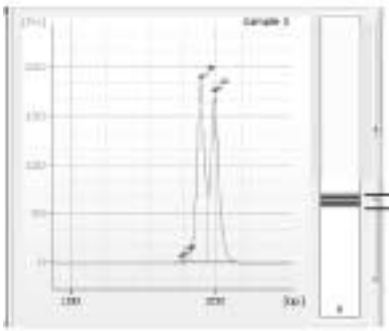
Conclusion

Relapse of the underlying disease continues to be the main cause of failure of allogeneic HSCT in acute leukemia. Thus, improvement in molecular monitoring of donor haematopoietic cells in the early post-transplantation period using high resolution microchip electrophoresis could be useful in predicting the occurrence of increasing recipient chimerism which may herald impending relapse in patients; while the disease burden is still low. This investigational approach may provide useful information for clinicians to select appropriate intervention strategies to ensure successful transplantation. However, validation with more cases needs to be done.



Pre tx

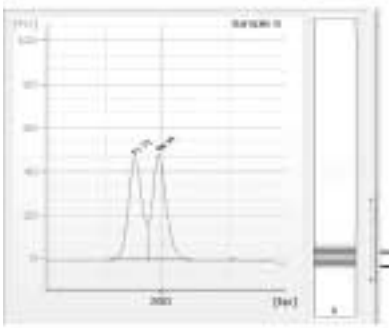
A^{rd} = 199 bp
A^{d} = 192



Donor

A^{rd} = 199 bp
A^{d} = 192 bp

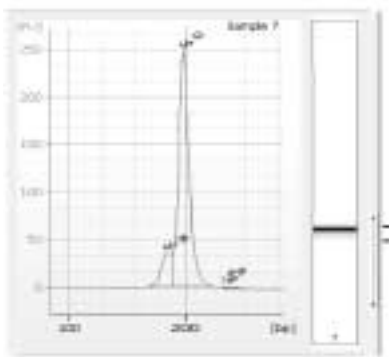
$$100 \times \left[\frac{2 \times A^{d}}{A^{d} + A^{rd}} \right] = \text{\% donor chimerism}$$



Post tx + 22

A^{rd} = 199 bp [FU: 0.97]
A^{d} = 192 bp [FU: 0.94]

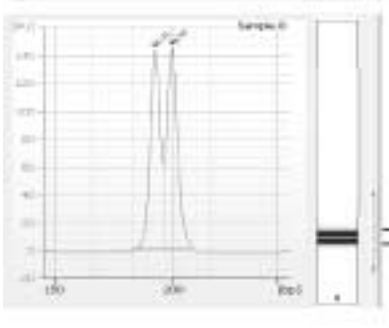
$$100 \times \left[\frac{2 \times 0.94}{0.94 + 0.97} \right] =$$ 98% donor chimerism



Post tx + 3 months

A^{rd} = 199 bp [FU: 4.82]
A^{d} = 192 bp [FU: 0.65]

$$100 \times \left[\frac{2 \times 0.65}{0.65 + 4.82} \right] =$$ 24% donor chimerism



Post tx + 4 months

A^{rd} = 199 bp [FU: 2.96]
A^{d} = 192 bp [FU: 3.03]

$$100 \times \left[\frac{2 \times 3.03}{3.03 + 2.96} \right] =$$ 100% donor chimerism

Figure 3. Schematic representation of patient MW percent donor chimerism calculation. Electropherogram next to the virtual gel image shows informative donor and recipient alleles of TH01STR locus. Peak area of donor specific allele is symbolised = A^{d} and peak area shared by both recipient and donor is symbolised = A^{rd}. The formula to calculate chimerism is based on Nollet *et al*, 2001[15]

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References

1. Sparkes R. Cytogenetic analysis in human bone marrow transplantation. Cancer Genet Cytogenet 1981; 4:345-52.
2. Schaap N, Schattenberg A, Bar B, *et al*. Red blood cell phenotyping is a sensitive technique for monitoring chronic myeloid leukemia patients after T-cell depletion bone marrow transplantation and after donor lymphocyte infusion. Br J Haematol 2000; 108:116-25.
3. Seong CM, Giralt S, Kantarjian H, *et al*. Early detection of relapse by hypermetaphase fluorescence in situ hybridisation after allogeneic bone marrow transplantation for chronic myeloid leukemia. J Clin Oncol 2000; 18:1831-36.
4. Lawler M, Humphries P, McCann S. Evaluation of mixed chimerism by in vitro amplification of dinucleotide repeat sequences using the polymerase chain reaction. Blood 1991; 77: 2504-14.
5. Suttorp M, Schmitz N, Dreger P, *et al.* Monitoring of chimerism after allogeneic bone marrow transplantation with unmanipulated marrow by use of DNA polymorphisms. Leukemia 1993; 7:679-87.
6. Sambrook J, Russell DW. Molecular Cloning: A Laboratory Manual. 3rd Ed. New York: Cold Spring Harbor Laboratory Press, 2001.
7. Choi SJ, Lee KH, Lee JH, *et al*. Prognostic value of hematopoietic chimerism in patients with acute leukemia after allogeneic bone marrow transplantation: a prospective study. Bone Marrow Transplant 2000; 26:327-32.
8. Diez-Martin JL, Gomez-Pineda A, Serran D, *et al* Successful treatment of incipient graft rejection with donor leukocyte infusions, further proof of a graft versus host lymphohaemopoietic effect. Bone Marrow Transplant 2004; 33:1037-41.
9. McSweeney PA, Storb R. Mixed chimerism: preclinical studies and clinical applications. Biol Blood Marrow Transplant 1999; 5:192-203.
10. Bader P, Niethammer D, Willasch A, *et al*. How and when should we monitor chimerism after allogeneic stem cell transplantation? Bone Marrow Transplant 2005; 35:107-19.
11. Barrios M, Jimenez-Velasco A, Roman-Gomez J, *et al*. Chimerism status is a useful predictor of relapse after allogeneic stem cell transplantation for acute leukemia. Haematologica 2003; 88: 801-10.
12. Bertheas MF, Lafage M, Levy P, *et al.* Influence of mixed chimerism on the results of allogeneic bone marrow transplantation for leukemia. Blood 1991; 78(11):3103-6.
13. Kamel AM, Mossalam G, Mahmaoud HK, *et al*. Variable number of tandem repeat polymorphism as a tool of chimerism detection in allogeneic stem cell transplantation. J Egyptian Nat Cancer Inst 2002; 14:275-87.
14. Mueller O. High resolution DNA analysis with the DNA 500 and DNA 1000 LabChip® kits. Application. Agilent Technologies 2001; 1:1-4.
15. Nollet F, Billiet J, Selleslag D, *et al.* Standardisation of multiplex fluorescent short tandem repeat analysis for chimerism testing. Bone Marrow Transplant 2001; 28:511-8.