Evaluation of two short tandem repeat multiplex systems for post-haematopoietic stem cell transplantation chimerism analysis

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ABSTRACT

Introduction: The follow-up of chimerism status after allogeneic haematopoietic stem cell transplantation (HSCT) is essential to predict successful engraftment to assess the development of graft-versus-host disease, graft rejection and disease relapse. Analysis of short tandem repeats (STR) via polymerase chain reaction is frequently used for chimerism determination. However, most commercially-available kits have been designed for forensic purposes and may not be optimal for chimerism analysis. The present study aims to identify suitable STR markers for patient-donor pairs of predominantly Malay and Chinese ethnicity using two commercially-available forensic kits.

Methods: We analysed six STR loci, namely, CSF1PO, TPOX, TH01 (using the CTT multiplex system) and F13A01, FESFPS and vWA (using the FFv multiplex system) in 33 human leukocyte antigen-matched Malaysian patient-donor pairs to determine the suitability of these two multiplex systems for chimerism analysis in our local population.

Results: Informativity (different alleles in donor and recipient) of each individual locus was TH01 73 percent, vWA 73 percent, F13A01 52 percent, CSF1PO 61 percent, FESFPS 39 percent and TPOX 45 percent. When combined, the six STR loci were able to give chimerism results in 31 out of 33 (94 percent) cases.

Conclusion: We found that TH01 and vWA are informative STR targets for post-HSCT chimerism analysis in predominantly Malay and Chinese patient-donor pairs. The commercially-available kits will also permit laboratories without extensive molecular biology capabilities to perform DNA typing in HSCT recipients.

Keywords: chimerism, microsatellites, polymerase chain reaction, short tandem repeats, stem cell transplantation

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INTRODUCTION

Haematopoietic stem cell transplantation (HSCT) is now an important mode of treatment for various haematological and metabolic disorders. Chimerism analysis following allogeneic HSCT is essential to predict successful engraftment. Although engraftment can be inferred from the recovery of peripheral blood cell counts, rising cell numbers may not necessarily mean that full donor chimerism has been attained. In addition, increasing recipient chimerism can be predictive of relapse and consequently the failure of the transplant. The use of T-cell depletion, non-myeloablative conditioning or novel graft versus host disease prophylactic regimens would require rapid and accurate serial chimerism analysis; because interventions such as donor lymphocyte infusion (DLI) may be required to convert mixed to complete donor chimerism.[1]

Different methods have been developed to monitor chimerism. Most of these methods make use of polymorphic markers to differentiate between donor and recipient cells. Early studies relied on techniques such as red blood cell phenotyping, cytogenetics, fluorescence in situ hybridisation (FISH) and polymerase chain reaction (PCR) amplification of variable number of tandem repeats (VNTR) (10–30 base pairs). Limitations of these studies include limited degrees of polymorphism, low sensitivity and a requirement for a large number of cells. FISH application is restricted to sex mismatched donor-recipient pairs.[2]

Short tandem repeats (STR) consist of repetitive sequences of three to seven base pairs of DNA.[3] They often occur in the untranslated parts of known genes

(whose sequence can be used as the PCR primers). The exact number of repeats varies in different people and therefore, PCR amplification will result in PCR products of different lengths depending on the number of the tandem repeats.[4-6] Currently, DNA-based technologies are the methods of choice for chimerism analysis, mainly due to their sensitivity in detecting the presence of a minor clone of recipient cells after transplantation.[7]

For forensic applications, STR loci should possess numerous observed alleles, a high level of heterozygosity, high polymorphism information content, and a high power of exclusion because the primary goal is individual identification.[8] As most HSCT are performed between siblings, they have a higher tendency to inherit the same alleles at a specific locus. Thus, informativity in the context of chimerism analysis is substantially different from that in the forensic field. The degree of heterozygosity, and not just the total number of alleles present, is important in analysing patient-donor chimerism.

The present study aims to identify suitable STR markers for patient-donor pairs of predominantly Malay and Chinese ethnicity using two commercially-available forensic kits. Commercially-available kits are now widely used for multiplex and also monoplex STR amplification and genotyping. The usage of these STR kits eliminates the troubleshooting process of thermal cycling programmes and ensures quality control of primers since these have been optimised. Commercially-available STR kits include AmpFlSTR® Identifiler™, Profiler Plus™ and COfiler™ from Applied Biosystems (Foster City, CA) and the GenePrint® STR Powerplex and GenePrint® Fluorescent Monoplex STR Systems from Promega Corporation (Madison, WI).

The primer sets included in these kits usually have been optimised to co-amplify six and up to 12 loci simultaneously in a single tube. However, the loci included in these kits have all been chosen based on profiles of African Americans, Hispanic Americans and Caucasians. In this study, we sought to evaluate two STR kits (using not more than six loci), which would be adequate for chimerism analysis in Malaysian HSCT patient-donor pairs. Loci chosen were those known to have a high power of discrimination and heterozygosity in the Malaysian population, as previously published by Panneerchelvam et al[8] and Seah et al.[9]

METHODS

33 patient-donor pairs were studied. Ethnic composition of the patients was Chinese (26), Malay (5) and Indian (2). 28 patients treated by allogeneic HSCT between April 2005 and June 2006 underwent chimerism analysis performed on Day 14, Day 30 and Day 60 post-HSCT. Another five patients were analysed as their previous VNTR studies were uninformative. This study was approved by the Medical Ethics Committee of the University of Malaya Medical Centre (MEC429.11/2005). DNA from 3–5 ml of peripheral blood collected in ethylenediaminetetraacetic acid tubes of patients and donors before and after HSCT was extracted using the modified phenol chloroform method, as previously described.[10] The extracted DNA was re-suspended in sterile double distilled water and kept at 4°C, until required. Purity and concentration was estimated from the optical density ratio (OD_{260}/OD_{280}) with a reading of between 1.7 and 1.9.

Table I. STR characteristics and multiplex system.

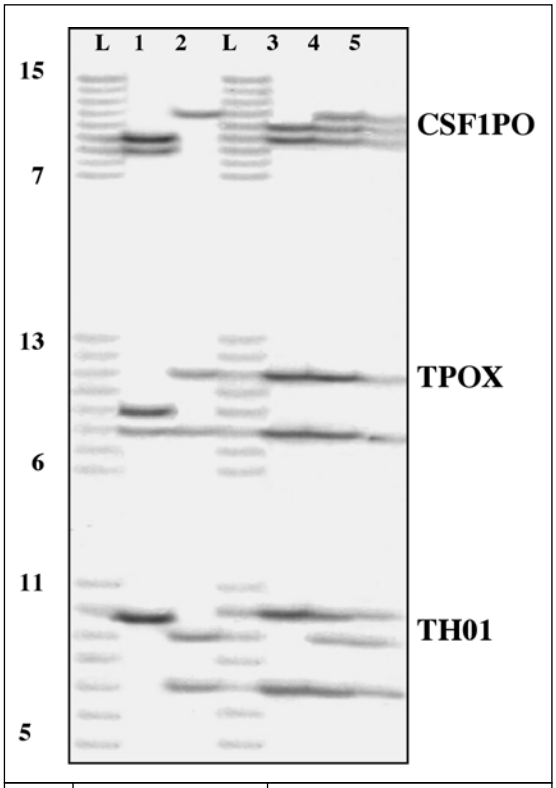
STR locus	Allelic ladder size range (bp)	Number of repeat units (alleles)	Repeat sequence 5'à3'
CSF1PO	295–327	7, 8, 9, 10, 11, 12, 13, 14, 15	AGAT
TPOX	224–252	6, 7, 8, 9, 10, 11, 12, 13	AATG
TH01	179–203	5, 6, 7, 8, 9, 10, 11	AATG
F13A01	283–331	4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16	AAAG
FESFPS	222–250	7, 8, 9, 10, 11, 12, 13, 14	AAAT
vWA	139–167	13, 14, 15, 16, 17, 18, 19, 20	AGAT

Multiplex STR analysis was performed using the GenePrint™ CTT and FFv Multiplex STR Systems (Promega, Madison, WI, USA). The CTT kit simultaneously amplifies three autosomal tetranucleotide STR loci: CSF1PO, TPOX and TH01 with non-overlapping allele size ranges while the FFv kit amplifies F13A01, FESFPS and vWA loci. Details of these loci are given in Table I. PCR was carried out in a final volume of 25 µL with 17.35 µL sterile nuclease free water, 100 ng genomic DNA sample, 2.5 µL 1× STR buffer, 2.5 µL 1× CTT multiplex primer pair mix (Promega, Madison, WI, USA) and 0.15 µL of HotStar Taq™ DNA polymerase (Qiagen, Hilden, Germany) using a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). 5 ng of cell line K562 DNA served as a positive amplification control to validate the result. The PCR cycling conditions consisted of initial denaturation at 96°C for two minutes, followed by ten cycles of denaturation at 94°C for one minute, annealing at 64°C for one minute and elongation at 70°C for 1.5 minutes. The PCR was then continued for an additional 20 cycles of denaturation at 90°C for one minute, annealing at one minute and elongation at 70°C for 1.5 minutes.

Thermocycling conditions for the amplification of FFv loci were similar to that used for the CTT loci with the exception of the annealing temperature, which was changed to 60°C and an additional extension step at 60°C

for 30 minutes. PCR products were analysed on a TVS 1000 DNA sequencing unit (Scie-Plas, England) with a set of 0.35 mm melinex spacers and a sharktooth comb using 4% (19:1) acrylamide:bisacrylamide denaturing (7 M urea) gel electrophoresis. A 3 µL portion of PCR product was mixed with 2.5 µL STR 2× loading solution containing 95% formamide. Samples were denatured at 95°C for two minutes and chilled immediately on ice. After a pre-run at 1500V for 30 minutes, 3 µL of sample mixture were loaded into respective wells. The process of loading did not take longer than 20 minutes to prevent the gel from cooling and following this, electrophoresis was carried out at 2,500V for 1.5 hours. Allelic ladders were loaded next to the sample lanes to facilitate assignment of alleles of each locus.

Following electrophoresis, the gel was subjected to silver staining for genotype determination. The gel was fixed in a solution containing 10% acetic acid and stained with a solution containing silver nitrate and formaldehyde for 30 minutes. Subsequently, the gel was rinsed and developed in an ice-cold alkaline sodium carbonate solution containing formaldehyde and sodium thiosulphate. After staining, the DNA fragments were viewed directly within the gel with the aid of a white light box. A permanent copy of the gel was obtained by scanning the gel using a desktop scanner. Chimerism post-HSCT was determined qualitatively by the presence of donor or recipient-specific bands (Fig. 1). The presence of donor bands solely in a recipient's blood suggested full chimerism while presence of both donor and recipient-specific bands implied a mixed chimerism.



Lanes	Samples	Component loci		
		CSF1PO	TPOX	TH01
1	K562 DNA	9, 10	8, 9	9.3, 9.3
2	Patient pre-treatment	12, 12	8, 11	7,9
3	Donor	10, 11	8, 11	7, 10
4	Patient Day 14	10, 11, 12	8, 11	7, 9, 10
5	Patient Day 30	10, 11, 12	8, 11	7, 9, 10

Fig. 1 Representative STR data on silver-stained gel of a patient following cord blood transplantation.The genomic DNA samples (lanes 1–5) were amplified using CTT multiplex. Presence of both donor and recipient-specific alleles in post-HSCT samples indicate a mixed chimerism state.
L: allelic ladder for the respective loci

RESULTS

The distribution of the polymorphisms in the 33 donor-recipient pairs was studied. To be informative, alleles from one locus of the recipient should differ from those of the donor. With regard to informativeness, 94% of the patients in our series had their engraftment status successfully analysed. Two pairs were non-informative for all six loci. STR markers were also found to be informative in the five archival patient samples in which VNTR was not successful. In the CTT multiplex, TH01 was the most informative locus where nearly three-quarters of the cases studied showed unique bands for donor and recipient. In the FFv multiplex kit, vWA was

Table II. Number of informative markers for chimerism analysis in 33 Malaysian HSCT patients using the CTT multiplex and FFv multiplex kits.

	STR locus	Total D/R pairs tested	No. of D/R pairs with informative alleles	Locus informativity (%)
CTT multiplex	CSF1PO	33	20	61
	TPOX	33	15	45
	TH01	33	24	73
FFv multiplex	F13A01	33	17	52
	FESFPS	33	13	39
	vWA	33	24	73

D/R: donor/recipient

the most informative locus. Either one or both TH01 and vWA STR loci were found to be informative in 30 patients. The informativeness for each STR marker is shown in Table II.

DISCUSSION

Our main aim was to investigate the best STR markers that can be used in our Malaysian population to determine engraftment status following HSCT. Developing an optimal method for detecting full chimerism may enhance the ability to identify patients at risk for early relapse.[11] Clearly, any method leading to early detection of graft loss is crucial, since it is now possible to prevent increasing mixed chimerism at an early stage with DLI. We have found that this method can detect mixed chimerism and the presence of host cells when the total peripheral blood leukocyte count is as low as 0.1×10^9/L. Therefore, engraftment status can be determined from a very early post-transplant period. Usage of allelic ladders in the analysis of STR system allows more accurate assignment of alleles. Components of the allelic ladder and the sample fragments have the same length and the same sequence and they will migrate the same distance during electrophoresis regardless of the types of the environmental changes.[12] This principle allows precise allele assignment for all our donor-recipient genotypes.

In contrast to forensic studies, for successful post-HSCT chimerism analysis, the degree of heterozygosity, and not just the total number of alleles present, is important. Not all STR loci that are routinely used as markers in forensic work, linkage studies and building genetic maps are suitable for chimerism purposes. For certain STR markers, the heterozygosity and power of discrimination (PoD) values may be exclusive and distinct from their informativity values when tested using the same origin of reference population. To illustrate this point, polymorphism studies done in ethnic Chinese in Malaysia have shown that loci FESFPS and TPOX have PoD and heterozygosity values of > 80%.[13] However, FESFPS and TPOX primer sets were found to give informative polymorphisms in only 50% and 46% of our 26 Chinese donor-recipient pairs, respectively. These two loci overall gave the lowest informative values and this suggests that they are not useful chimerism markers. Thus, informativity in the context of chimerism analysis is substantially different from that in the forensic field.

The methodology described in this study is a qualitative method and is known to be imprecise. However, to evaluate a graft's performance at different time intervals after transplantation and to enable clinical decisions to be made, quantitation is always feasible whenever there is accessibility to a fluorescence imaging system such as capillary electrophoresis, Genetic Analyzer® (Applied Biosystems, Foster City, CA, USA) or a DNA sequencer.[14] The Genescan® electropherogram programme can be used to produce an estimate of the quantity of DNA of the STR alleles. We hope the information from this pilot STR analysis using real patients undergoing HSCT is useful before choosing a candidate STR locus for quantitative chimerism analysis, especially in the Asian population.

As STR-based human identity kits are commercially available in various multiplex assays and are frequently used in forensic laboratories, the need for developing the methodology in each individual laboratory is eliminated.[15] However, we feel that it is not cost effective to test a total of nine or 12 STR loci, as most commercial multiplex kits have all these primers pre-mixed into a single solution. For post-transplant chimerism analysis, even one informative STR locus is already adequate to be used for quantitative analysis. A set of two or three STR primers (with known high informative values) chosen to form an STR panel is more reasonable than amplifying a set of nine loci but having half of the primer sets giving non informative results. In our cohort, if we had only used both TH01 and vWA STR loci for initial screening, we would have been able to successfully perform chimerism analysis in 30 out of 33 (91%) patients as either one or both loci have been found to be informative.

In conclusion, by using a panel of six different STR markers scattered on different chromosomes, we were able to determine engraftment status in 13 adults and 18 children who underwent HSCT. STR genotyping is independent of HLA mismatching or sex mismatching and can be used immediately after transplant when very few cells are available for analysis. We were also able to show successful adaptation of commercial forensic STR reagent kits for monitoring chimerism in post-HSCT patients. It is reasonable to consider the information presented in this study as a step before recruiting suitable loci to be used for quantitative chimerism analysis. Further studies to identify optimal STR markers for individual ethnic groups would be useful.

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