Bone Marrow Transplantation (2006) 37, 751–755

www.nature.com/bmt

ORIGINAL ARTICLE

Developing an algorithm of informative markers for evaluation of chimerism after allogeneic bone marrow transplantation

S Sellathamby, P Balasubramanian, S Sivalingam, RV Shaji, V Mathews, B George, A Viswabandya, A Srivastava and M Chandy

Department of Haematology, Christian Medical College, Vellore, India

Analysis of chimerism by polymerase chain reaction amplification of STR or VNTR has become a routine procedure for the evaluation of engraftment after allogeneic stem cell transplantation. Knowledge of the frequency of different STR or VNTR alleles in unrelated individuals in a population is useful for forensic work. In the context of HLA identical sibling bone marrow transplantation the informativeness of these markers needs to be evaluated. We evaluated five STRs (THO1, VWA, FES, ACTBP2, and F13A1) and 1 VNTR (APOB) for informativeness in stem cell transplants from HLA identical sibling donors. All four markers used individually allowed us to discriminate 20–56% of the patient donor pairs. Using a combination of all these markers along with a polymorphic marker in the β-globin gene and the sex chromosome specific amelogenin marker, we were able to discriminate 99% of the patient donor pairs. We have established an algorithm for evaluating chimerism following HLA identical sibling donor transplants in the Indian population using molecular markers in 310 patients. Analysis of heterozygote frequencies in different populations is similar suggesting that this algorithm can be used universally for transplant centers to evaluate chimerism following allogeneic bone marrow transplantation.
Bone Marrow Transplantation (2006) **37,** 751–755. doi:10.1038/sj.bmt.1705317; published online 6 March 2006
Keywords: informativeness; chimerism; STRs

Introduction

Short tandem repeat (STR) or variable number tandem repeat (VNTR) loci are groups of DNA sequences, which are highly polymorphic markers and can therefore be used for identification of an individual. They are used for a variety of applications including forensic identification,[1] phylogenetic reconstruction[2] and to monitor chimerism after allogeneic bone marrow transplantation for early diagnosis of graft failure or disease relapse. Allelic frequencies, allelic distribution pattern and level of heterozygosity of certain microsatellite markers has already been reported in various ethnic groups. A microsatellite locus used for chimerism analysis must have a different allelic profile in the patient and donor. Several studies have shown that the overall informativeness of STRs differs significantly between related and unrelated individuals.[3] There are limited data on the informativeness of various VNTRs or STRs in HLA identical recipient donor pairs. Many transplant centers use STR kits to monitor chimerism but these are quite expensive. We screened five STRs namely FES, THO1, VWA, ACTBP2, and F13A1 and 1 VNTR, APOB, in order to develop an algorithm of sequential testing for evaluation of chimerism in a cost effective manner after allogeneic stem cell transplantation.

Patients and methods

Chimerism was studied retrospectively in 310 hematopoietic stem cell transplants (HSCT) during the period 1993–2005. All donors were HLA identical siblings or related family members. Two hundred and ninety five (95%) patients and donors were studied by polymerase chain reaction (PCR) for one or more of the polymorphic repeat sequences given in the Table 1 but for 15 (5%), which were sex, mismatched only the amelogenin PCR was performed. The number of patients evaluated with THO1 was 263 (89%), ACTBP2: 265 (90%), FES: 248 (84%) and VWA: 255 (86%). Combined data on informativeness of all these four markers was available for 219 patients (74%). In this study, 293 patients were from India: 120 (39%) from the South, 88 (28%) from the North, 25 (8%) from the East and 59 (19%) from the West of India. Seventeen (5%) of the patients were from other countries: Maldives ($n=4$), Oman ($n=4$), Sri Lanka ($n=2$), Singapore ($n=1$), Mauritius ($n=1$), Pakistan ($n=3$), and Bhutan ($n=2$). A STR/VNTR marker was considered to be informative if any one of the alleles of the patient is different from that of the donor. Parents were not genotyped and therefore a pedigree analysis for these STR/VNTRs is not possible.

Correspondence: Dr M Chandy, Department of Haematology, Christian Medical College, Vellore 623 004, India.
E-mail: mammen@cmcvellore.ac.in
Received 19 September 2005; revised 22 December 2005; accepted 20 January 2006; published online 6 March 2006

Table 1 Gene location of different microsatellite and minisatellite markers

Marker	*Satellite type*	*Gene name*	*Repeat sequences*	*Chromosome location*
FES	Micro	Human c-fes proto-oncogene	(ATTT) *n*	15q25-qter
THO1	Micro	Tyrosine hydroxylase gene	CATT/(AATG) *n*	11p15–15.5
VWA	Micro	von Willebrand antigen gene	(TCTA) *n*	12p12-pter
ACTBP2	Micro	Actin, beta pseudogene 2	(AAAG) *n*	6q14
APOB	Mini	Apolipoprotein gene	TTTTATAATTAATATTTATAAT	2p24–p23
F13A01	Mini	Human coagulation factor XIII a subunit gene	(AAAG) *n*	6p24.3–25.1

Table 2 Primer sequences for satellite markers

Marker	*Primer sequences (Sequences in parenthesis are GC clamps)*	*Fluorochrome label*
FES[4]	F: 5′ GGG ATT TCC CTA TGG ATT GG 3′ R: 5′ GCG AAA GAA TGA GAC TAC AT 3′	Hex
THO1[5]	F: 5′ GTG GGC TGA AAA GCT CCC GAT TAT 3′ R: 5′ GTG ATT CCC ATT GGC CTG TTC CTC 3′	FAM
ACTBP2[6]	F: 5′ AAT CTG GGC GAC AAG AGT GA 3′ R: 5′ ACA TCT CCC CTA CCG CTA TA 3′	Hex
VWA[7]	F: 5′ AGC TAT ATA TAT CTA TTT ATC AT 3′ R: 5′ AGA TAC ATA CAT AGA TAT AGG 3′	FAM
APOB	F: 5′ GAA ACG GAG AAA TTA TGG AGG G 3′ R: 5′ TCC TGA GAT CAA TAA CCT CG 3′	Nil
F13A1[5]	F: 5′ ATG CCA TGC AGA TTA GAA A 3′ R: 5′ GAG GTT GCA CTC CAG CCT TT 3′	Hex
AMG	A: 5′ CTG ATG GTT GGC CTC AAG CCT GTG 3′ B: 5′ TAA AGA GAT TCA TTA ACT TGA CTG 3′ C: 5′ GCC CAA AGT TAG TAA TTT TAC 3′	Nil
G-fragment β-globin gene[8]	F: 5′ (GCC CGC CGT CCC GGC CCG ACC CCC GCG CGT CCG GCG CCC G) CTG GGC ATG TGG AGA CAG AG 3′ R: 5′ CAC TGA TGC AAT CAT TCG TC 3′	Nil

DNA analysis

DNA was extracted using a standard extraction protocol. Both patient and donor samples were amplified by PCR with specific primer pairs for the five STRs.[4–7] The marker, gene, and chromosomal location is given in Table 1. Primer sequences for the above-mentioned markers are given in Table 2. Polymerase chain reaction was performed using standard concentrations of primers (0.3 μM) and 2 × PCR master mix (ABgene®, Blenheim Road, UK) containing 0.2 mM of each dNTPs, 20 mM $(NH_4)_2SO_4$, 75 mM Tris HCl (pH 8.8 at 25°C, 1.5 mM $MgCl_2$, 0.01% (v/v) Tween 20 and 1.25 U Taq DNA polymerase. Polymerase chain reaction conditions for ACTBP2, FES, THO1, VWA, and F13A1 markers were initial denaturation at 94°C for 5 min, followed by 30 cycles with denaturation at 94°C for 40 s and annealing and extension at 57°C for 2 min and 72°C for 5 min, respectively. Polymerase chain reaction products were analyzed in 10 or 12% polyacrylamide gel. Polymerase chain reaction for APOB marker was performed with the annealing temperature of 58°C for 5 min. Amelogenin PCR for amplifying a region on X and Y chromosome was performed using 1 × buffer, 0.25 mM dNTPs, 0.4 μm of each primers, 0.5 μM of $MgCl_2$ and 1 U Amersham Taq polymerase for a single reaction at an annealing temperature of 62°C for 2 min. Polymerase chain reaction products were analyzed in 2% agarose gel.

Temporal temperature gradient gel electrophoresis. A fragment of β-globin gene (IVS-II) containing three intragenic polymorphisms (nucleotide (nt) 16, C/G; nt 74, G/T; and nt 81, C/T) was amplified by PCR using the primer sequences[8] given in Table 2. Polymerase chain reaction products were analyzed in 6% polyacrylamide (acrylamide: bis 37.5:1) gels containing 6 mol/l urea in a temperature gradient. Electrophoresis was carried out at 130 V at constant temperature increments of 2°C/h on the D Code TM mutation detection system (Bio-Rad Laboratories, Hercules, CA, USA), which has an automatic thermal regulator that increases temperature gradually over the length of electrophoresis.

Genescan. Polymerase chain reaction products obtained using fluorescence labeled primers were mixed with 12 μl of deionized formamide and 0.5 μl of Rox 500 size standard and denatured at 95°C for 5 min. These samples were subjected to denaturing capillary gel electrophoresis using ABI 310 sequencer (Applied Biosystems, Foster city, CA, USA) Data were analyzed using the Genotyper 2.5 software. The proportion of patient and donor in post-BMT samples was calculated as previously described.[9]

Results

All four markers used individually allowed us to discriminate 44–56% of the patient donor pairs. Of the four markers, THO1 (56%) and ACTBP2 (53%) were found to be highly informative, followed by VWA (50%), and FES (44%). By combining one, two or three markers, we could discriminate between 68 and 92% of the patient donor pairs. Informativeness of the markers THO1 + VWF,

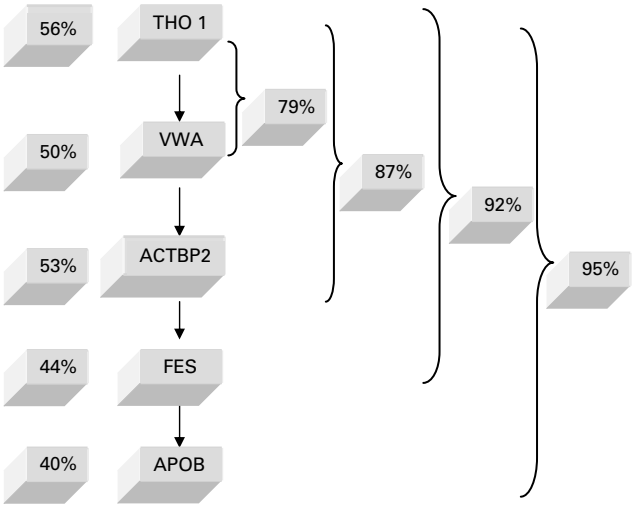

Figure 1 Schematic representation of informativeness of the STRs/VNTRs used in our cohort. Adding a Genescan method along with β-globin gene and amelogenin markers to the panel of screening informative marker has allowed us to discriminate 99% of patient donor pairs in our cohort.

THO1 + VWF + ACTBP2, THO1 + VWF + ACTBP2 + FES was 79, 87, and 92%, respectively. None of these markers was informative in 18 patients. APOB was informative in six of these 18 patients (33%) and for the whole group it was informative in 40% (52/131).

We have recently added a new marker, F13A1, into our panel and it was the only informative marker in one patient. Schematic representation of informativeness of the STRs/VNTRs used in our cohort is shown in Figure 1.

Of the remaining 11 patients, two were sex-mismatched transplants and hence amelogenin PCR was used for documenting the chimerism status. In the remaining nine patients, the G-fragment of the β-globin gene was analyzed by temporal temperature gradient gel electrophoresis (TTGE) and this allowed us to discriminate two cases. Thus, the above panel was informative in 98% of the transplants.

In the seven patients where no informative marker could be identified, gene scan analysis was useful in five patients. Thus, this panel of molecular markers was informative for chimerism analysis in 308 of 310 (99%) allogeneic stem cell transplants.

There were 166 sex-mismatched transplants and of these 115 were female to male transplants where the amelogenin gene PCR is useful for chimerism analysis. Data on the amelogenin gene PCR were available for 43 of these patients and 24 showed mixed chimerism. In 11 of these 24 patients, analysis with one of the other STRs showed complete chimerism suggesting that the amelogenin PCR is a more sensitive indicator of the chimeric status.

Discussion

Monitoring of chimerism after allogeneic bone marrow transplantation is important for early diagnosis of graft failure or disease relapse. Use of DNA-based diagnostics by STRs is the most suitable method for monitoring chimerism post transplant.[10–15]

Published data on sequential testing of chimerism and informativeness of different markers is limited.[16–19] Spyridonidis *et al*.[16] using a combination of five STR markers (SE 33, THO1, D1S80, YNZ22, and D14S120) found this panel to be informative in 98% of 165 related transplants. A similar study from north India has reported 100% informativeness using five STRs (VWA, THO1, FES, F13, and TPOX).[17] A recent study suggests that a similar panel can be used both for related and unrelated donor transplants.[18] Grubic *et al*.[19] were able to discriminate nine HLA identical sibling patient donor pairs using a panel of STRs contaning 6–10 markers. The panel of STR/VNTR markers used in this study was based on the heterozygosity value in Caucasoid population. Thiede *et al*.[18] have showed a linear correlation of heterozygosity with the number of highly informative STR constellations, which are well suited for chimerism analysis.

In our panel, THO1 was found to be the most informative (56%) and F13A1, the least (20%). When none of these markers in our panel were informative, polymorphisms in the G-fragment of β-globin gene and the Y chromosome specific amelogenin marker proved to be useful.

Table 3 compares the data from north India with this study[17] and Caucasian data which shows some difference in informativeness of individual markers,[18] which can be attributed to ethnic variations.

Microsatellites are prone to mutation during replication and this has implications when they are used for paternity analyses or disease linkage mapping, where a mutation may lead to false exclusion. Mutation rates have been found to be different between various loci and among alleles of a particular locus, depending on the repeat structure of the microsatellite. Reported mutational rates for the markers used in this study are: THO1-0%, FES-0.117%, VWA-0.199%, and ACTBP2-0.684% per locus per gamete per generation.[20] However, gametic mutation in an STR will not interfere with its usefulness in chimerism analysis.

For sex-mismatched transplants the Y and X chromosome specific amelogenin PCR has a reported sensitivity of one male cell in 10^6 female cells.[21] A Y chromosome specific band was detected post transplant in 11 female to male transplants when the other markers suggested that the chimerism was complete, confirming the higher sensitivity

Table 3 Comparison of informativeness in HLA identical siblings

Markers	*Our study % informativeness*	*North Indian study (n = 42) (%)*	*Thiede* et al.[18] *(n = 203) % informativeness*
THO1	56	55	65*
VWA	50	62	67***
F13A1	20*	44	—
FES	44	55	—
ACTBP2	53	—	68**

*$P > 0.01$; **$P > 0.001$; ***$P > 0.0001$.

Table 4 Average heterozygosity of markers from different population

Markers	*Africans*	*Caucasians*	*Caucasoids*	*Orientals*
F13A1	0.73145	0.75748	0.6366	0.6436
APOB	0.8181	0.7579	0.7014	0.5868
ACTBP2	0.9135	0.94375	0.91475	0.9353
FES	0.75175	0.66336	0.7543	0.6585
THO1	0.69662	0.77359	0.687	0.6805
VWA	0.7528	0.7611	0.758	0.7825
AH	0.7773	0.77619	0.742	0.71453

References for the heterozygosity of various STRs and VNTRs.
http://www.uni-duesseldorf.de/WWW/MedFak/Serology/database.html.
http://www.cstl.nist.gov/div831/strbase/str_ref.htm.

of the amelogenin PCR. However, sensitivity of a marker and its correlation with clinical outcome needs to be evaluated further.

If either the patient or the donor has a unique band on PAGE analysis, genescan can be used to evaluate chimerism and this was useful in six patients, increasing the informative value of the same STR markers from 95 to 98% in this study.

Quantification of the level of chimerism is necessary for serial monitoring and this can be performed by newer techniques like FISH, STR/VNTR genescan analysis and real-time PCR. Although FISH has a high sensitivity of 0.1–0.001%, it can be used only for sex-mismatched transplants. Recently Buno *et al*. have demonstrated that STR-PCR is better than XY-FISH for quantifying chimerism and is independent of the sex mismatch.[22] Highly sensitive quantitative real-time PCR (detection limit – 0.001–0.0001) has also been applied for monitoring chimerism, however, the SNPs to be used and the clinical significance of documenting mixed chimerism at a very low level needs to be evaluated.[23]

Chimerism assessment with capillary electrophoresis by fluorescence-based PCR amplification of STRs is currently the most suitable method for post transplant chimerism with a sensitivity of 1–5% and high accuracy[24] but formation of stutter peaks and preferential amplification of the shorter allele are limitations. Highly specific STR amplification by TD-PCR by Fundia *et al*.[25] has further improved the utility of di-nt STRs in post-BMT chimerism analysis.

Short tandem repeat PCR and conventional PAGE followed by capillary electrophoresis for quantification is cost effective (£28–30 for the first post-BMT screening and £8.4 for subsequent tests once the informative marker has been found) when compared to commercial kits (range £38–77). The average heterozygosity of all the markers used in this study is similar in most populations (Table 4) and therefore this algorithm of testing can be widely used for chimerism analysis post-BMT.

Acknowledgements

This study was supported in part by Grant BT/PRO 948/Med/13/034/98 from the Department of Biotechnology, Ministry of Science and Technology, Government of India.

References

1 Hammond HA, Jin L, Zhong Y, Caskey CT, Chakraborty R. Evaluation of 13 short tandem repeat loci for use in personal identification applications. *Am J Hum Genet* 1994; **55**: 175–189.
2 Rowold DJ, Herrera RJ. Inferring recent human phylogenies using forensic STR technology. *Forensic Sci Int* 2003; **133**: 260–265.
3 Bryant E, Martin PJ. Documentation of engraftment and characterization of chimerism following hematopoietic cell transplantation. In: Thomas ED, Blume KG, Forman SJ (eds). *Hematopoietic Cell Transplantation*. Blackwell Science: Oxford, London, 1999, pp 197–206.
4 Polymeropoulos MH, Rath DS, Xiao H, Merril CR. Tetranucleotide repeat polymorphism at the human c-fes/fps protooncogene (FES). *Nucleic Acids Res* 1991; **19**: 4018.
5 Kimpton CP, Gill P, Walton A, Urquhart A, Millican ES, Adams M. Automated DNA profiling employing multiplex amplification of short tandem repeat loci. *PCR Meth Appl* 1993; **3**: 13–22.
6 Polymeropoulos MH, Rath DS, Xiao H, Merril CR. Tetranucleotide repeat polymorphism at the human beta-actin related pseudogene H-beta-Ac-psi-2 (ACTBP2). *Nucleic Acids Res* 1992; **20**: 1432.
7 Peake IR, Bowen D, Bignell P, Liddell MB, Sadler JE, Standen G *et al*. Family studies and prenatal diagnosis in severe von Willebrand disease by polymerase chain reaction amplification of a variable number tandem repeat region of the von Willebrand factor gene. *Blood* 1990; **76**: 555–561.
8 Ghanem N, Girodon E, Vidaud M, Martin J, Fanen P, Plassa F *et al*. A comprehensive scanning method for rapid detection of beta-globin gene mutations and polymorphisms. *Hum Mutat* 1992; **1**: 229–239.
9 Schichman SA, Suess P, Vertino AM, Gray PS. Comparison of short tandem repeat and variable number tandem repeat genetic markers for quantitative determination of allogeneic bone marrow transplant engraftment. *Bone Marrow Transplant* 2002; **29**: 243–248.
10 Ginsburg D, Antin JH, Smith BR, Orkin SH, Rappeport JM. Origin of cell populations after bone marrow transplantation. Analysis using DNA sequence polymorphisms. *J Clin Invest* 1985; **75**: 596–603.
11 Knowlton RG, Brown VA, Braman JC, Barker D, Schumm JW, Murray C *et al*. Use of highly polymorphic DNA probes for genotypic analysis following bone marrow transplantation. *Blood* 1986; **68**: 378–385.
12 Thein SL, Jeffreys AJ, Blacklock HA. Identification of post-transplant cell population by DNA fingerprint analysis. *Lancet* 1986; **2**: 37.

13 Lawler M, Humphries P, McCann SR. Evaluation of mixed chimerism by *in vitro* amplification of dinucleotide repeat sequences using the polymerase chain reaction. *Blood* 1991; **77**: 2504–2514.
14 Ugozzoli L, Yam P, Petz LD, Ferrara GB, Champlin RE, Forman SJ *et al*. Amplification by the polymerase chain reaction of hypervariable regions of the human genome for evaluation of chimerism after bone marrow transplantation. *Blood* 1991; **77**: 1607–1615.
15 van Leeuwen JE, van Tol MJ, Bodzinga BG, Wijnen JT, van der Keur M, Joosten AM *et al*. Detection of mixed chimaerism in flow-sorted cell subpopulations by PCR-amplified VNTR markers after allogeneic bone marrow transplantation. *Br J Haematol* 1991; **79**: 218–225.
16 Spyridonidis A, Zeiser R, Wasch R, Bertz H, Finke J. Capillary electrophoresis for chimerism monitoring by PCR amplification of microsatellite markers after allogeneic hematopoietic cell transplantation. *Clin Transplant* 2005; **19**: 350–356.
17 Suraksha A, Khan F, Sudha T, Sonia N. Short tandem repeat technology has diverse applications: Individual identification, Phylogenetic reconstruction and chimerism based post haematopoietic stem cell transplantation graft monitoring. *Indian J Med Sci* 2004; **58**: 297–303.
18 Thiede C, Bornhauser M, Ehninger G. Evaluation of STR informativity for chimerism testing – comparative analysis of 27 STR systems in 203 matched related donor recipient pairs. *Leukemia* 2004; **18**: 248–254.
19 Grubic Z, Stingl K, Cecuk-Jelicic E, Zunec R, Serventi Seiwerth R, Labar B *et al*. Evaluation of mixed chimerism in bone marrow transplantation program in Croatia. *Transplant Proc* 2005; **37**: 1388–1391.
20 Brinkmann B, Klintschar M, Neuhuber F, Huhne J, Rolf B. Mutation rate in human microsatellites: influence of the structure and length of the tandem repeat. *Am J Hum Genet* 1998; **62**: 1408–1415.
21 Pugatsch T, Oppenheim A, Slavin S. Improved single-step PCR assay for sex identification post-allogeneic sex-mismatched BMT. *Bone Marrow Transplant* 1996; **17**: 273–275.
22 Buno I, Nava P, Simon A, Gonzalez-Rivera M, Jimenez JL, Balsalobre P *et al*. A comparison of fluorescent *in situ* hybridization and multiplex short tandem repeat polymerase chain reaction for quantifying chimerism after stem cell transplantation. *Haematologica* 2005; **90**: 1373–1379.
23 Alizadeh M, Bernard M, Danic B, Dauriac C, Birebent B, Lapart C *et al*. Quantitative assessment of hematopoietic chimerism after bone marrow transplantation by real-time quantitative polymerase chain reaction. *Blood* 2002; **99**: 4618–4625.
24 Bader P, Niethammer D, Willasch A, Kreyenberg H, Klingebiel T. How and when should we monitor chimerism after allogeneic stem cell transplantation? *Bone Marrow Transplant* 2005; **35**: 107–119.
25 Fundia AF, De Brasi C, Larripa I. Feasibility of a cost-effective approach to evaluate short tandem repeat markers suitable for chimerism follow-up. *Mol Diagn* 2004; **8**: 87–91.