VNTR Markers for Qualitative Evaluation of Engraftment in Unrelated Cord Blood Transplantations

S M Noor, MMedSc*, M E Phipps, PhD*, M Y Fong, PhD, L L Chan FRCP*****

*Department of Molecular Medicine, **Department of Parasitology, ***Department of Paediatrics, Faculty of Medicine, University of Malaya, Kuala Lumpur

SUMMARY

Allogeneic stem cell transplantation is a treatment option for malignant and non-malignant disorders in children. For children with no HLA-matched sibling or related stem cell donors, there is the option of unrelated cord blood donors. At the University of Malaya Medical Centre (UMMC) in Kuala Lumpur, the first unrelated cord blood transplantation (CBT) was performed in October 1997. All unrelated CBT performed in UMMC relied on cord blood units imported from overseas. DNA typing with variable number of tandem repeat (VNTR) loci was done to qualitatively evaluate engraftment in 15 unrelated CBT. In all the fifteen cases that were evaluated, molecular evidence of engraftment or non-engraftment correlated with the clinical findings.

KEY WORDS:
DNA typing, VNTR, Engraftment, Unrelated cord blood transplantation

INTRODUCTION

Since the first cord blood transplantation (CBT) in 1988,[1] CBT has become a viable alternative to bone marrow transplantation (BMT), especially in paediatric patients. Cord blood is one of three sources of haematopoietic stem cells for transplantation (the other two being the bone marrow and peripheral blood), and of the three, cord blood offers the lowest risk of graft-versus-host disease (GVHD), thanks to the immaturity and naïvity of cord blood lymphocytes[2,3]. The criteria for HLA matching between donor and recipient can thus be less stringent, which eases the burden of procuring an unrelated donor[4]. Another advantage is that cord blood units are mostly free from cytomegalovirus infection, which has been reported to contribute to a high mortality risk amongst stem cell transplant recipients[5].

Cord blood is collected from the umbilical cord of a newborn; the blood, rich in haematopoietic stem cells, is cryopreserved and stored until such time when the blood may be thawed for use in transplantation[6]. Cord blood banks are now established worldwide and there are registries such as Netcord (http://www.netcord.org) and Bone Marrow Donors Worldwide (BMDW; http://www.bmdw.org) that allow fast searches for matching donor units, which, once identified, can be transported to the transplant centre immediately for use[7,8,9]. Malaysia's own public cord blood bank is still in its initial growth stage, so for the time being patients in Malaysia who require unrelated cord units are dependent on the import of foreign cord blood units.

From October 1997 to September 2005, thirty-six CBT were performed in the University of Malaya Medical Centre (UMMC). The majority of the CBT patients relied on their siblings for cord blood stem cell donation. Unrelated CBT were carried out with donor units imported from cord blood banks in Australia, Europe, the USA, Japan, Taiwan, and Singapore. The unrelated cases involved children between the ages of 8 months to 12 years, with no HLA-matched sibling donors. The patients' diagnoses included acute leukaemia, juvenile myelomonocytic leukaemia, Wiskott-Aldrich Syndrome, β-thalassaemia major, and osteopetrosis.

Following transplantation, it is vital that the patient engrafts successfully. Engraftment indicates that donor stem cells have begun haematopoiesis in the patient's marrow, leading to the successful reconstitution of the patient's blood cell counts and immune functions. Adversely, engraftment could be followed by GVHD, which requires vigilant post-transplant monitoring and treatment[10,11]. A fine balance has to be maintained between engraftment and avoidance of GVHD, and knowing with certainty that a patient exhibiting GVHD-like symptoms has engrafted can help clinicians formulate the proper course of action to save both the graft and the patient. Likewise, proper response can be planned for a patient who shows no signs of recovery past the time engraftment would be expected. This is especially important in an unrelated CBT which would have already incurred a high financial cost by this point.

The time to haematologic recovery following transplantation is one yardstick by which engraftment is ascertained. A neutrophil count of $> 1.0 \times 10^9$/litre, maintained over three consecutive days, and an untransfused platelet count of $> 50 \times 10^9$/litre, also maintained over three consecutive days would be the first indications of haematologic recovery. However, as welcome as haematologic recovery is, it does not in itself rule out autologous recovery. There must be ways to determine that there has been engraftment of the donor's cells.[12,13] In

This article was accepted: 1 January 2007
Corresponding Author: Suzita Mohd Noor, Biomedical Science Programme, Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

sex-mismatched transplantations, cytogenetic typing for the sex chromosome provides engraftment information, and blood group markers are used in cases where there is major ABO-mismatch between donor and recipient, provided there has not been any recent transfusion.

With the advent of molecular techniques and polymerase chain reaction (PCR), we can now determine engraftment by identifying a patient's post-transplant haematologic cells to be of donor origin, independent of gender and blood groups[14, 15]. VNTR are variable number of tandem repeats – minisatellites: sequences of about 15 – 50 base pairs (bp) in length that randomly repeat in tandem at hypervariable regions in the human genome. These tandemly repeating sequences offer polymorphisms which can be amplified by PCR to produce a unique profile, a DNA 'fingerprint'[16]. No two individuals, save identical twins, will share the same genetic profile.

Applying this method of identification to the stem cell transplantation scenario, the patient and his or her donor would, to begin with, have distinguishing and unique genetic profiles. Following stem cell transplantation, a patient who has engrafted would be expected to exhibit a haematologic molecular profile identical to the donor's – recognised as full chimaerism (Figure 1), or as an intermediate between that of the patient's own and the donor's: a state known as mixed chimaerism (Figure 2), whereby cells of different origin exist together within the same individual.

MATERIALS AND METHODS

Between the years 2000 to 2005, fifteen unrelated CBT cases at the UMMC Paediatric Bone Marrow Transplant Unit were evaluated for molecular signs of engraftment. Peripheral blood or marrow samples were collected from recipients prior to transplant. The samples were collected in EDTA tubes and stored frozen.

Cord blood donor stem cells were received as frozen units in cryobags, whereby the cord blood had been volume reduced, cryopreserved in 10% DMSO, and stored in liquid nitrogen (average sample temperature maintained at -155°C). On the day of transplantation, cord blood unit bags were removed from liquid nitrogen and thawed in a 37°C water bath. Thawed stem cells were then infused directly to the recipient. Donor samples for analyses were obtained by rinsing the empty bag with 1.0 – 3.0 ml of normal saline. On occasion, a separate 1.5 ml cryovial of cryopreserved donor stem cells in 10% DMSO would be provided by the foreign cord blood bank, and these preserved vial cells would be used for DNA extraction.

Post-transplant peripheral blood samples were collected when the recipient's nucleated cell count had recovered to more than 1.0 x 10^9/L. One recipient never achieved the desired nucleated count, and for this recipient, peripheral blood samples were nonetheless collected on Day 15, then again on Day 30. The recipient died 39 days post-transplant without ever achieving a white blood cell count of more than 1.0 x 10^9/L.

DNA was extracted by previously established methods[17]. Polymerase chain reaction was done using primers for D1S80[18] and D17S30[19], and the amplicons were separated by polyacrylamide gel electrophoresis (PAGE). Gels were stained with ethidium bromide and visualised under ultraviolet light. Amplicons were visualized and documented on the GelDoc platform. Profiles of patient and recipient pairs were observed and discriminated; in the event that there was discrimination between recipient pre-transplant and donor, the next step was then to identify the match between the post-transplant recipient and donor profiles.

RESULTS

For all fifteen cases, unique profiles were obtained for every individual, allowing for informative discrimination between respective recipient and donor pairs. Engraftment was

Table I: Transplant case information and transplant outcome, both molecular and clinical, within months after transplantation, and recipients' current clinical condition.

Recipient	Diagnosis	Origin of CB unit	Day WBC exceeded 1.0x10^9/L	Molecular Outcome	Clinical Outcome of Donor Graft	Clinical Condition as of December 2005	Cause of Death
1	CML	Melbourne	41	No Engraftment	Did not engraft	Alive with disease	-
2	WAS	Milan	32	Engraftment	Engrafted	Alive and well	-
3	JML	Sydney	36	Engraftment	Engrafted	Died on day 241	Acute renal failure
4	ALL	Dusseldorf	28	Engraftment	Engrafted	Died on day 93	Infection & GVHD
5	JML	San Diego	29	Engraftment	Engrafted	Died on day 43	Multi-organ failure & GVHD
6	AML	Taiwan	29	Engraftment	Engrafted	Alive and well	-
7	WAS	Taiwan	24	Engraftment	Engrafted	Alive and well	-
8	JML	Taiwan	27	No Engraftment	Did not engraft	Died on day 84	JML
9	ALL	Taiwan	53	No Engraftment	Did not engraft	Died on day 165	ALL
10	ALL	Tokyo	25	Engraftment	Engrafted	Died on day 56	GVHD
11	Osteo	New York	-	No Engraftment	Did not engraft	Died on day 39	Pulmonary haemorrhage
12	Osteo	Taiwan	35	No Engraftment	Did not engraft	Died on day 92	Underlying disease
13	β-Thal	Taiwan	17	Engraftment	Engrafted	Alive and well	-
14	β-Thal	Taiwan	15	Engraftment	Engrafted	Alive and well	-
15	β-Thal	Taiwan	19	No Engraftment	Did not engraft	Alive with disease	-

Abbreviations for Table I

ALL – Acute Lymphoblastic Leukaemia; AML – Acute Myeloblastic Leukaemia;
CML – Chronic Myeloid Leukaemia; JML – Juvenile Myeloblastic Leukaemia;
WAS – Wiskott-Aldrich Syndrome; Osteo – Osteopetrosis; β-Thal – β-Thalassaemia Major;
WBC – White blood cells; GVHD – Graft-versus-Host Disease

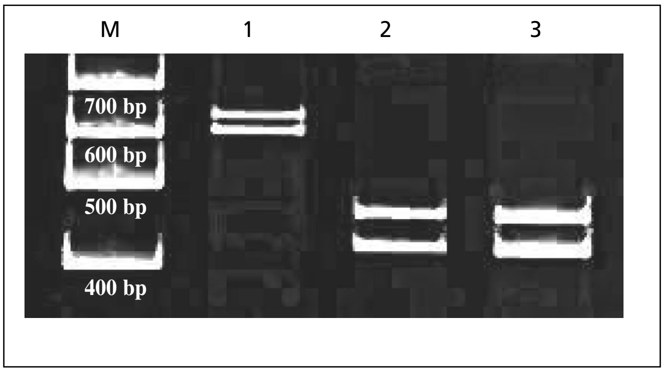

Fig. 1: Representative PAGE of observable molecular profiles:
(1) Recipient/patient prior to transplant;
(2) The donor;
(3) The recipient after transplant - profile identical to donor's.

M = 100 bp molecular weight marker

The recipient's pre-transplant and donor respective profiles are easily discriminated, and after transplantation, the recipient's donor-like profile is seen.

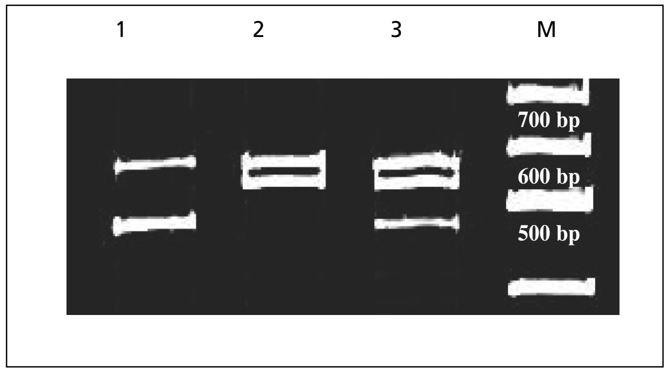

Fig. 2: Representative PAGE of observable molecular profiles:
(1) Recipient/patient prior to transplant;
(2) The donor;
(3) The recipient after transplant - profile is a mix of the donor and recipient pre-transplant.

M = 100 bp molecular weight marker

The recipient's pre-transplant and donor respective profiles are easily discriminated, and after transplantation, the recipient profile is that of a chimaera.

inferred when the recipient's post-transplant DNA profile completely matched the donor's, or was a mixed chimaeric pattern of the donor's and the recipient's pre-transplant amplicons. In cases of non-engraftment, the recipient's post-transplant profile remained unchanged from the pre-transplant profile, with no presence of donor-like amplicons.

All fifteen cases yielded early post-transplant molecular profiles that led to the inference of either engraftment or non-engraftment, eventually correlating with and supporting clinical observations made in these cases (summarised in Table I). Of the fifteen, VNTR typing showed nine children had engraftment, while six had not. The six who showed no molecular engraftment were clinically determined to have experienced autologous recovery, correlating with the observation that the non-engrafted recipients' post-transplant molecular profiles remained unchanged from their pre-transplant profiles.

The recipient whose blood counts never recovered to more than 1.0 x 10^9/L had nonetheless had a Day 15 typing done, which revealed non-engraftment. When VNTR typing was done on a Day 30 sample, the result was still that of non-engraftment. The recipient died nine days later.

Although all donor DNA were extracted from thawed stem cells that had been cryopreserved in 10% DMSO, no interferences were seen in the PCR output of these samples, and donor VNTR profiles remained consistent when PCR was repeated.

DISCUSSION AND CONCLUSION

Molecular evaluation of engraftment was achieved with fair accuracy once informative DNA profiles were obtained that discriminated between the recipient and his or her donor. In our case, two VNTR markers provided informative discrimination for each unrelated recipient-donor pair, and post-transplant engraftment status could be successfully inferred.

With PCR permitting reliable amplification of thousands of copies of the respective D1S80 and D17S30 VNTR sequences, analyses could be performed even with low amounts of DNA. This is a matter of great importance, for the volume of the imported donor cord unit would be sufficient only for infusion to the recipient, leaving very little for sampling and DNA extraction. However, since cord blood is rich in nucleated stem cells, DNA could be extracted even from small volumes of donor cells which were obtained either directly in a vial from the cord blood bank supplying the donor unit, or by rinsing the cord unit bag with saline after the stem cells had been thawed and infused to the patient.

The results obtained from the VNTR typing of unrelated CBT recipients and their donors offered a quick answer to the question of whether the recipient was looking at a successful post-unrelated cord blood transplantation outcome. While VNTR polymorphisms served to effectively distinguish the molecular profiles of the recipient from the donor in each of these reported cases, there are other better techniques now perfected that could provide results with a greater degree of confidence. There are limitations to how many donor/recipient pairs can be distinguished and discriminated by VNTR typing, especially when the SCT patient and donor are siblings. Additional DNA markers in the form of short tandem repeats (STRs) [20, 21] provide a higher degree of polymorphism because of their shorter length, which increases the likelihood of successfully discriminating between recipient and donor profiles [22]. The availability of commercial multiplex assay STR-based human identity kits greatly eases the process of analysis, besides offering the capability of running more samples simultaneously.

Indeed, with the right optimizations, STR typing is the method of choice for future developments in the molecular determination of chimerism [23, 24] and is in fact the technique that the UMMC is currently employing, with the eventual aim of establishing the more desirable method of quantitatively determining engraftment, which could be provided by real time PCR[25].

ACKNOWLEDGEMENTS

This study was supported in part by Vote F (F0150/2001A). Many thanks go to Dr. Lin Hai Peng, the PI BMT ward of UMMC, and the Department of Parasitology, Faculty of Medicine, UM.

REFERENCES

1. Gluckman E, Broxmeyer HE, Auerbach AD *et al*. Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical cord blood from an HLA-identical sibling. N Engl J Med 1989; 321: 1174-8.
2. Madrigal JA, Cohen SBA, Gluckman E *et al*. Does cord blood transplantation result in lower graft versus host disease? It takes more than two to tango. Hum Immunol 1997; 56(1-2): 1-5.
3. Rocha V, Wagner JE, Sobocinski KA *et al*. Graft versus host disease in children who have received a cord blood or bone marrow transplant from an HLA-identical sibling. N Engl J Med 2000; 342: 1846-54.
4. Wagner JE, Barker JN, DeFor TE *et al*. Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival. Blood 2002; 100(5): 1611-8.
5. Nichols WG, Corey L, Gooley T *et al*. High risk of death due to bacterial and fungal infection among cytomegalovirus (CMV)-seronegative recipients of stem cell transplants from seropositive donors: evidence for indirect effects of primary CMV infection. J Infect Dis 2002; 185: 273-82.
6. Broxmeyer HE, Douglas GW, Hangoc G *et al*. Human umbilical cord blood as a potential sourse of transplantable haematopoietic stem/progenitor cells. Proc Natl Acad Sci USA 1989; 86(10): 3828-32.
7. Miniero R, Rocha V, Saracco P *et al*. Cord blood transplantation (CBT) in hemoglobinopathies. Eurocord. Bone Marrow Transplant 1998; 22 (Suppl 1): S78-9.
8. Krishnamurti L, Abel S, Maiers M, *et al*. Availability of unrelated donors for hematopoietic stem cell transplantation for hemoglobinopathies. Bone Marrow Transplant 2003; 31(7): 547-50.
9. Davey S, Armitage S, Rocha V *et al*. The London Cord Blood Bank: analysis of banking and transplantation outcome. Br J Haematol 2004; 125(3): 358-65.
10. Wagner JE, Rosenthal J, Sweetman R *et al*. Successful transplantation of HLA matched and HLA mismatched umbilical cord blood from unrelated donors: analysis of engraftment and acute graft versus host disease. Blood 1996; 88(3): 795-802.
11. Thomson BG, Robertson KA, Gowan D *et al*. Analysis of engraftment, graft-versus-host disease, and immune recovery following unrelated donor cord blood transplantation. Blood 2000; 96(8): 2703-11.
12. Khan F, Agarwal A, Agrawal S. Significance of chimerism in hematopoietic stem cell transplantation: new variations on an old theme. Bone Marrow Transplant 2004; 34: 1-12.
13. 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.
14. Ugozzoli L, Yam P, Petz LD *et al*. Amplification by polymerase chain reaction of hypervariable regions of the human genome for evaluation of chimerism after bone marrow transplantation. Blood 1991, 77(7): 1607-15.
15. Sreenan JJ, Pettay JD, Tbakhi A *et al*. The use of amplified variable number of tandem repeats (VNTR) in the detection of chimerism following bone marrow transplantation. A comparison with restricted fragment length polymorphism (RFLP) by Southern blotting. Am J Clin Pathology 1997; 107: 292-8.
16. Jeffreys AJ, Wilson V, Thein SL *et al*. DNA 'fingerprints' and segregation analysis of multiple markers in human pedigrees. Am J Hum Genet 1986; 39: 11-24.
17. Bloom MV, Freyer GA, Micklos DA (eds). Laboratory DNA Science. Menlo Park, California: Benjamin/Cummings Pub. Co., 1995.
18. Kasai K, Nakamura Y, White R. Amplification of a variable number of tandem repeats (VNTR) locus (pMCT118) by the polymerase chain reaction (PCR) and its application to forensic science. J Forensic Sci 1990; 35(5): 1196-200.
19. Nakamura Y, Ballard L, Leppert M *et al*. Isolation and mapping of a polymorphic DNA sequence (pYNZ22) on chromosome 17p (D17S30). Nucleic Acids Res 1988; 16(12): 5707.
20. Jeffreys AJ, Wilson V, Thein SL. Individual-specific 'fingerprints' of human DNA. Nature 1985; 316(6023): 76-9.
21. Fregeau CJ, Fourney RM. DNA typing with fluorescently tagged short tandem repeats: a sensitive and accurate approach to human identification. Biotechniques 1993; 15(1): 100-19.
22. Schichman SA, Suess P, Vertino AM *et al*. 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(3): 243-8.
23. 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(2): 87-91.
24. 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(2): 248-54.
25. Koldehoff M, Steckel NK, Hlinka M *et al*. Quantitative analysis of chimerism after allogeneic stem cell transplantation by real-time polymerase chain reaction with single nucleotide polymorphisms, standard tandem repeats, and Y-chromosome-specific sequences. Am J Hematol 2006; 81(10): 735-46.