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, 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 naivety of cord blood lymphocytes. The criteria for HLA matching between donor and recipient can thus be less stringent, which eases the burden of procuring an unrelated donor. 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.

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. 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. 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. 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 x 10⁹/litre, maintained over three consecutive days, and an untransfused platelet count of > 50 x 10⁹/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. In
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. 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 can be amplified by PCR to produce a unique profile, a DNA ‘fingerprint’. 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. Polymerase chain reaction was done using primers for D1S8018 and D17S3019, and the amplicons were separated by polyacrylamide gel electrophoresis (PAGE). Gels were stained with ethidium bromide and visualised under ultraviolet light. Amplicons were visualised 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>
<thead>
<tr>
<th>Recipient</th>
<th>Diagnosis</th>
<th>Origin of CB unit</th>
<th>Day WBC exceeded 1.0x10^9/L</th>
<th>Molecular Outcome</th>
<th>Clinical Outcome of Donor Graft</th>
<th>Clinical Condition as of December 2005</th>
<th>Cause of Death</th>
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<td>Milan</td>
<td>32</td>
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<td>ALL</td>
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<td>Did not engraft</td>
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</table>

**Abbreviations for Table I**

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) provide a higher degree of polymorphism because of their shorter length, which increases the likelihood of successfully discriminating between recipient and donor profiles. 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.
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Indeed, with the right optimizations, STR typing is the method of choice for future developments in the molecular determination of chimerism 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.

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