Quantitative Analysis of Chimerism after Allogeneic Peripheral Blood Stem Cell Transplantation

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Background: For peripheral blood stem cell transplantation (PSCT), several engraftment analysis methods have been performed including detection of restriction fragment length polymorphisms and amplification of polymorphic genetic loci. To facilitate monitoring of the engraftment, a quantitative, non-isotopic method using a short tandem repeat (STR) marker has been set up in our laboratory.

Methods: DNAs from pretransplant recipients and donors were amplified with the AmpFlSTR Profiler Plus kit that contains 9 STR markers. The fluorescent polymerase chain reaction products were then fractionated on polyacrylamide gels in an ABI PRISM 377 DNA sequencer. Results were analyzed using GeneScan 2.1 software. We selected the best markers as informative alleles which can distinguish donor from recipient. For quantitative analysis of the engraftment, we prepared a mixed chimeric sample by mixing pretransplant recipient and donor DNAs in different ratios to produce a standard curve. After amplifying the posttransplant recipient DNA, we were able to detect the extent of engraftment by interpolating the percent peak area of the informative alleles from this standard curve.

Results: We retrospectively analyzed 10 patients who had received allogeneic PSCT. Two of them showed some degree of mixed chimerism indicating leukemic relapse. In case one, 38.7% of the recipient DNA was first detected in the third month after PSCT. In case two, 6.5% of the recipient DNA was first detected in the tenth month after PSCT.

Conclusion: In summary, this method provides an accurate, quantitative, and early assessment of mixed chimerism in posttransplant patients. Such information may be useful to guide implementation of additional treatment to circumvent graft failure or relapse in the future.

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Key words: peripheral blood stem cell transplantation (PSCT), quantitative short tandem repeats (STRs), mixed chimerism.

At present, allogeneic peripheral blood stem cell transplantation (PSCT) is considered the best therapeutic option for patients with severe aplastic anemia, severe combined immunodeficiency disease,

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and leukemia. Detection of the extent of chimerism after transplantation is an important method for monitoring the engraftment of donor cells, thus allowing early detection of graft failure. In the past, clinical physicians used cytogenetic analysis or erythrocyte phenotyping to assess the extent of chimerism. Recently, several approaches have been published based on polymorphic DNA loci, such as restriction fragment length polymorphisms, variable number tandem repeats, and short tandem repeats (STRs).[1-3] The use of these markers not only allows an assessment of the extent of chimerism but also requires a very small amount of sample. Because STRs are interspersed throughout the genome and commercial applications are available, the use of STR loci has become a frequently used medical technology for the study of engraftment. However, the method is often limited by its ability to discriminate between donors and recipients. To facilitate the monitoring of peripheral blood stem cell engraftment, we developed a novel approach using a multiplex polymerase chain reaction (PCR) amplification of 9 STR loci and the amelogenin locus which provides a rapid, accurate, and quantitative way to determine the extent of chimerism after allogeneic PSCT.

METHODS

Patient samples

This retrospective study included 10 patients who received allogeneic PSCT at Chang Gung Memorial Hospital. Patient details are shown in Table 1.

Table 1. Patient Characteristics

PN	Diagnosis	Age (yr)	Stem cell	CD34/KG	Outcome
1	MDS/AML	23	Allo-PSCT	32 ¡ 10⁶	died, relapse
2	CML	18	Allo-PSCT	11 ¡ 10⁶	alive, well
3	CML	39	Allo-PSCT	23 ¡ 10⁶	alive, well
4	MDS	18	Allo-PSCT	9 ¡ 10⁶	died, relapse
5	CML	41	Allo-PSCT	25 ¡ 10⁶	alive, well
6	SAA	24	Allo-PSCT	14 ¡ 10⁶	alive, well
7	CML	40	Allo-PSCT	8 ¡ 10⁶	alive, well
8	SAA	20	Allo-PSCT	10 ¡ 10⁶	alive, well
9	SAA	25	Allo-PSCT	16 ¡ 10⁶	alive, well
10	AML	54	Allo-PSCT	12 ¡ 10⁶	alive, well

Abbreviations: PN: patient number; MDS: myelodysplastic syndrome; AML: acute myelogenous leukemia; CML: chronic myelogenous leukemia; SAA: severe aplastic anemia; Allo-PSCT: allogeneic peripheral blood stem cell transplantation.

Sample preparation

Peripheral blood samples were collected from a donor and recipient before allogeneic PSCT and from the recipient at regular intervals after the procedure. Genomic DNA was extracted from fresh blood samples using a QIAamp DNA mini kit (QIAGEN, Hidden, Germany).

Preparation of mixed chimeric DNA reconstructions

Before quantitative analysis of peripheral blood stem cell engraftment, we first had to prepare pre-PSCT recipient and donor DNAs in varying ratios of between 0% and 100% to produce a standard curve for every individual case. A reconstruction consisting of mixtures of varying percentages of pretransplant recipient and donor DNAs represented the degree of amplification of each allele in a mixed chimeric posttransplant sample. In these reconstructions, while the percentages of donor and recipient DNA changed relative to each other, the total amount of DNA in the reconstruction remained constant.

STR amplification

We used the AmpFlSTR Profiler Plus PCR amplification kit (Applied Biosystems, CA, USA) to perform STR-PCR. The tetranucleotide STR loci amplified in this reaction included: D3S1358, vWA, and FGA[4-6] (all labeled with 5-FAM); CSF1PO, TPOX, and TH01[7-9] (all labeled with JOE); and D5S818, D13S317, and D7S820[10,11] (all labeled with NED). In addition, the amelogenin locus was analyzed, which discriminates between X and Y chromosomes (labeled with JOE). For each PCR reaction, the final volume was 25 µl and included 5 ng of DNA as recommended by the manufacturer. The cycle conditions were: 95 °C for 11 min, followed by 28 cycles of 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min. The final elongation step was 45 min at 60 °C.

Electrophoresis and GeneScan analysis

The amplified PCR products were separated and detected using an ABI377 automated DNA sequencer (Applied Biosystems). A denaturing polyacrylamide gel containing 10X TBE, 6 M urea, and 5% Long Ranger gel solution (FMC Biosystems, Maine, USA) was used. For each sample, an internal size standard (GeneScan Rox 500, ABI, Warrington,

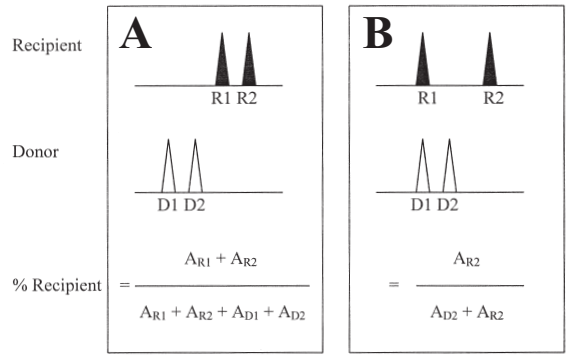

Fig. 1 Schematic illustration of calculations performed for the quantitative assessment of mixed chimerism. R1 and R2 are signals for recipient alleles 1 and 2; D1 and D2 are signals for donor alleles 1 and 2; A is the area of the specified peak signal. (A) Calculation when alleles of both the donor and recipient have distinct peaks. (B) Calculation in the situation of 2 heterozygous alleles, with 1 allele shared.

UK) and a formamide loading dye solution were added. After denaturation, 1.5 £gl of this mixture was loaded onto the gel, which was run for 2 h at 2.2 kV and 56 °C. Then the results were analyzed using the GeneScan 2.1 software.

Calculation of mixed chimerism

The recipient percentage peak area of the reconstruction samples was calculated and plotted versus the percentage of recipient DNA amplified to determine the engraftment of the posttransplant samples. The recipient percentage peak area was calculated as follows: %Peak area = $[(A_{R1}+A_{R2}) \times 100\%] / (A_{D1}+A_{D2}+A_{R1}+A_{R2})$,[12] where A is the peak area; D is the donor alleles; and R is the recipient alleles (Fig. 1). In cases of shared alleles, only the informative alleles (i.e., those that distinguish donor from recipient) were used in the calculation. We detected the extent of engraftment or relapse by interpolating the peak area of the informative alleles using this standard curve, and the engraftment or relapse was expressed as either percent donor or percent recipient DNA.

RESULTS

Precision, accuracy, and the standard curve

Because shorter alleles may amplify more efficiently than longer alleles, it is important to simulate a range of mixed chimerism that might be encountered in actual clinical posttransplant samples. We performed dilution experiments in order to test the linearity, reproducibility, precision, and sensitivity of the STR-PCR for quantitative assessment of mixed chimerism. Figure 2 shows the GeneScan data for the reconstruction samples in this experiment. The donor peak showed increasing donor allele peak area with increasing percentage of donor DNA. Conversely, the upper recipient allele peak area decreased as the percentage of recipient DNA decreased. The percent peak area data for the informative alleles of the reconstruction samples were calculated and used to plot a standard curve (Fig. 3). Although the donor peak area of the standard curve for each marker was directly proportional to the percent of donor DNA added, the curve for each locus varied slightly. Because amplification of the alleles differed from each other, this variation emphasizes the importance of preparing standard curves for the informative markers for each set of samples.

Sensitivity

In dilution experiments for plotting the standard curve, a minor cell population representing 5% was reproducibly detected. Even below 5%, signals could be found depending on the constellation of peaks present. In other words, alleles for which 1 individual was homozygous could be seen down to 1%. In addition to this, the use of multiple alleles increased the reliability of results, because of the higher chance of encountering an informative peak constellation.

Informative STR alleles

There were many combinations of donor and recipient alleles, and some of these combinations were informative for the detection of recipient alleles, whereas others were not. Although some loci were technically informative, they were not optimal for use in determining mixed chimerism because of the existence of stutter bands that appeared predominantly as 2 minor peaks alongside the major allele peaks. In general, stutter peaks are 1 repeat smaller or larger than the major allele peaks and typically 10% or less than the area of the major allele. They can influence the choice of informative loci for engraftment analysis.

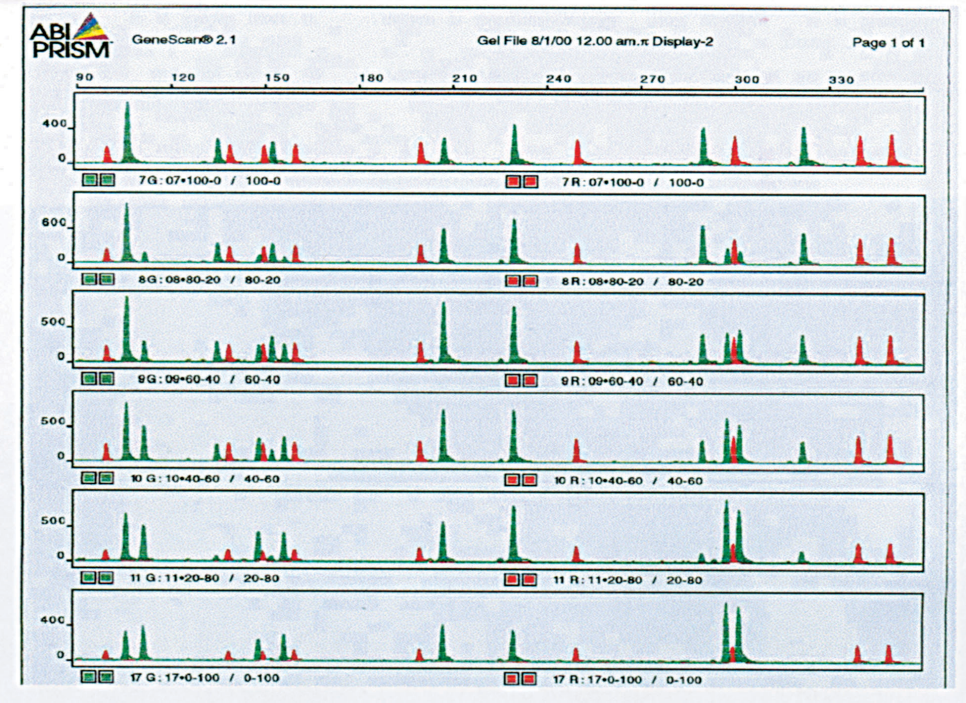

Fig. 2 GeneScan electropherogram of mixed chimeric reconstruction. The alleles shown, from left to right, are amelogenin, D8S1179, D21S11, and D18S51 labeled with the fluorophore JOE. The scale at the left of each electropherogram is the peak height. Lane 1: 100% recipient (R), 0% donor (D); lane 2: 80% R, 20% D; lane 3: 60% R, 40% D; lane 4: 40% R, 60% D; lane 5: 20% R, 80% D; lane 6: 0% R, 100% D.

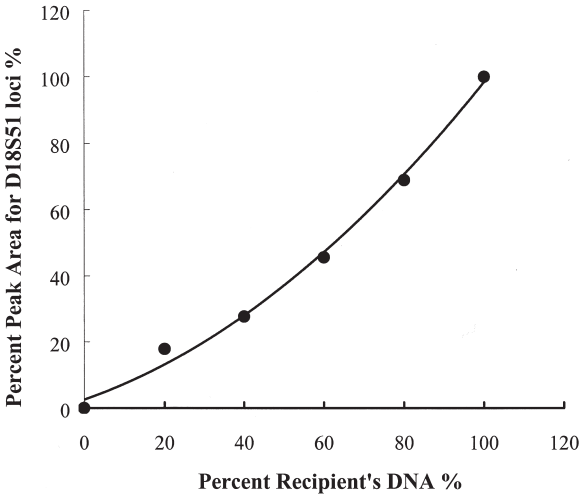

Fig. 3 Reconstruction standard curve for the informative locus of D18S51 as analyzed for peak area percent and recipient DNA percent. Goodness-of-fit: r2 = 0.997.

Clinical samples

Clinical DNA samples from the panel of 10 transplant patients' peripheral blood cells were amplified for STR markers. Information on the patients is given in Table 1. Figure 4 uses 1 of these patients as an example. Donor and patient pretransplant samples were found to be informative with D18S51. The D18S51 markers were sized and genotyped with the D18S51 allelic ladder from the Applied Biosystems AmpFlSTR Profiler Plus kit. Of the 10 patients examined, all patients showed complete engraftment after PBST, but the recipient DNA in 2 patients appeared to relapse after a period of time (Fig. 5). One recipient's DNA appeared at 3 months post-transplantation, while the other appeared after 10 months. In the first relapsed recipient, interpolation of the reconstruction plot shows that 27.82% of the recipient peak area of the post-transplant sample corresponded to 38.7% of the

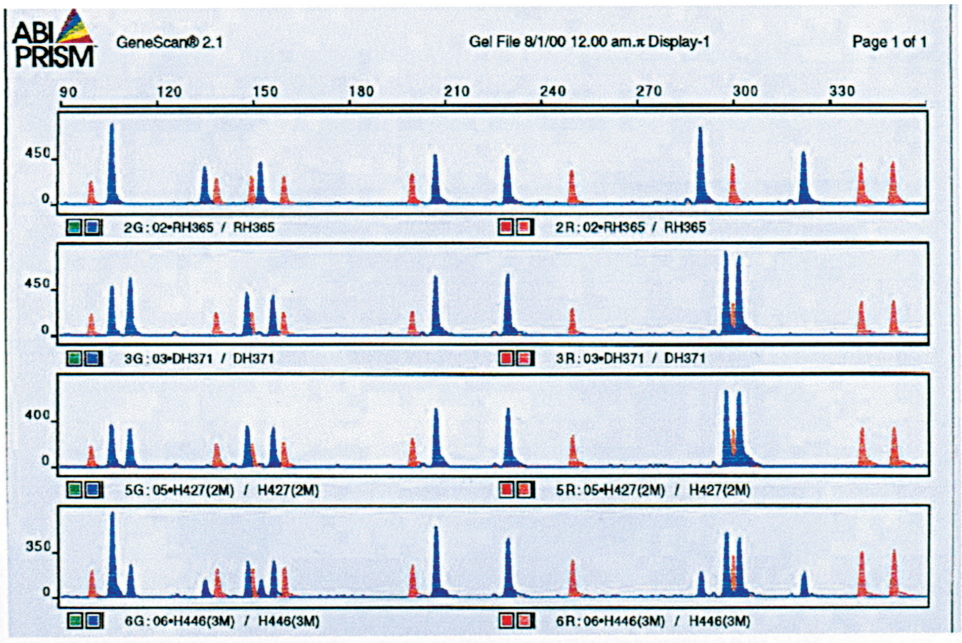

Fig. 4 GeneScan electropherogram of clinical sample UPN 1. The alleles shown, from left to right, are amelogenin, D8S1179, D21S11, and D18S51 labeled with the fluorophore JOE. Lane 1: recipient STR markers before transplantation; lane 2: donor STR markers; lane 3: recipient STR markers after 2 months; lane 4: recipient STR markers after 3 months. Some relapsed alleles appear in lane 4.

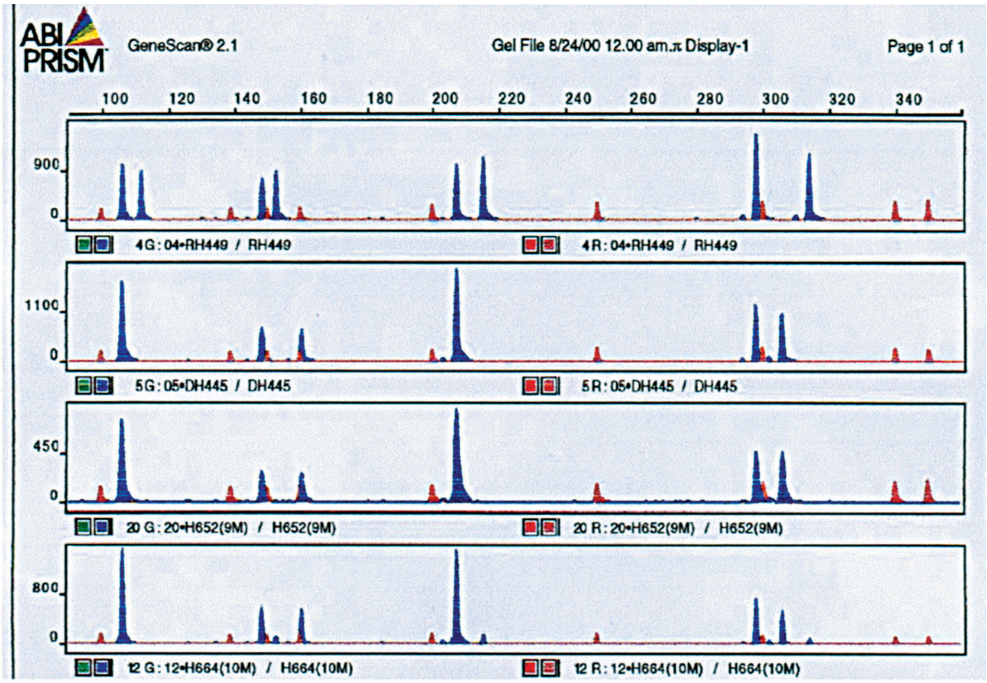

Fig. 5 GeneScan electropherogram of UPN 4. The alleles shown, from left to right, are amelogenin, D8S1179, D21S11, and D18S51 labeled with the fluorophore JOE. Lane 1: recipient STR markers before transplantation; lane 2: donor STR markers; lane 3: recipient STR markers after 9 months; lane 4: recipient STR markers after 10 months. Some relapsed alleles appear in lane 4.

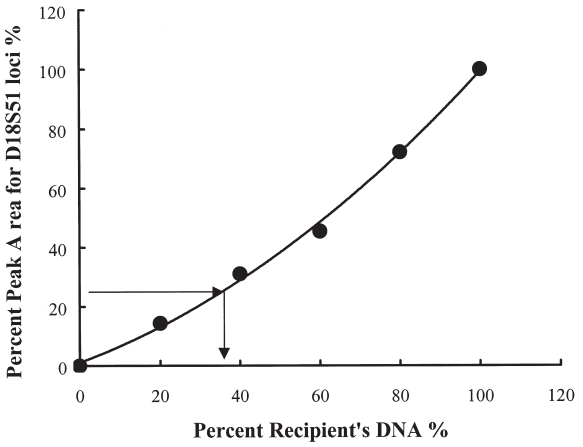

Fig. 6 Reconstruction standard curve for the informative locus of D18S51 of clinical sample UPN 1 as analyzed for peak area percent and recipient DNA percent. This figure shows that 27.82% of the recipient peak area of the posttransplant sample corresponds to 38.7% of the recipient DNA. Goodness-of-fit: r2 = 0.997.

recipient DNA (Fig. 6). The other showed that 1.67% of the recipient peak area of the posttransplant sample corresponded to 6.5% of the recipient DNA.

DISCUSSION

Peripheral blood stem cell transplantation has been established as a lifesaving procedure in selected hematologic malignancies and bone marrow failure syndromes, and it may be valuable in other types of neoplastic disease. Usually, relapse is a significant cause of transplantation failure. Because most relapses occur by expansion of a surviving leukemic clone of recipient origin, early detection of the progressive reappearance of recipient cells by bone marrow engraftment analysis may help to predict clinical relapse. For patients with relapse of chronic myelogenous leukemia, the effectiveness of adoptive immunotherapy by donor lymphocyte infusion to produce clinical remission has been well documented.[13-16] With acute leukemia, a second PSCT is a reasonable therapeutic approach for the treatment of a relapse. So it is necessary to carry out peripheral blood stem cell engraftment analysis of allogeneic PSCT patients to confirm the engraftment and to detect mixed chimerism or recipient relapse after transplantation.

DNA polymorphisms are used to monitor engraftment after transplantation from a related or unrelated donor. DNA polymorphisms are not useful after autologous BMT. If the donor is an identical twin, DNA polymorphisms are not useful, either. The most valuable polymorphism for this purpose is caused by variations in certain repeated sequences that are known as short tandem repeats (STRs). An informative STR locus is one for which at least 1 recipient allele has a different number of repeats than the donor allele(s). Because the recipient and donor are often related, they share many alleles. Therefore, multiple STR loci need to be tested to identify 1 or more informative loci.[17] The simultaneous amplification of multiple loci in a single reaction tube facilitates the identification of informative loci.

Although some loci are technically informative, they might not be optimal for the determination of mixed chimerism primarily because of the existence of stutter bands that appear predominantly as 2 minor peaks alongside the major allele peaks. The minor peaks are predominantly 1 repeat smaller or larger than the major allele peak. Stutter peaks have been proposed to result from slipped strand mispairing during PCR amplification,[18] which results in a peak that is 1 repeat smaller or larger than the major allele peak. The existence of stutter peaks influences the choice of informative loci for engraftment analysis. In a state of recipient DNA relapse, the percentage of recipient DNA is less than 5% to 10%, which is the same size as a typical stutter peak. Hence it is necessary to use the following criterion to choose the best locus: the recipient-specific allele must be at least 2 repeats larger or smaller than the nearest donor allele.

When an informative locus is selected, thee STR-PCR method can be used to qualitatively evaluate the engraftment.[3,12,19,20] A quantitative method for BME analysis using fluorescently labeled primers for PCR amplification of individual VNTR and STR loci was first described in 1995 by Scharf et al.[12] An electrical peak area for each end-labeled fragment detected is directly proportional to the number of DNA molecules present. To simulate a posttransplant MC or a relapsed state, we mixed 2 different DNA samples that displayed recipient and donor in various ratios of between 0% and 100% using the same total amount of 5 ng DNA to prepare the amplification reconstruction standard curves.

Because shorter alleles may amplify more efficiently than longer alleles, it is often not possible to use a simple visual estimation of the peak intensity to accurately determine the extent of mixed chimerism. In addition, it is critical to determine engraftment because of the potential for preferential amplification in mixtures of alleles. These preferential amplification effects depend on a variety of factors: the amount of DNA or enzyme used, the inherent efficiency of the amplification system, and the number and the relative size difference of the alleles present in the sample. We have found that the reconstruction standard curves obtained for a specimen can vary among different markers. In addition, different donor or recipient samples can produce different standard curves for the same marker. So it is very important to establish individual reconstruction standard curves for each marker in each donor-recipient couple.

From our experience in preparing standard curves, we found that the sensitivity of detection of small percentages of recipient DNA is related to the amount of genomic DNA in the PCR reaction. The use of 10 to 15 ng of DNA may improve the sensitivity of detecting small amounts of recipient cells by sampling more DNA, but this amount often results in nonspecific peaks that ultimately decrease the sensitivity by increasing the background, especially in multiplex reactions. The use of 1 to 3 ng of DNA generally gives a clean baseline above which recipient-specific peaks can easily be seen, but the sensitivity is lower. As a result of these findings, we routinely use 5 ng of DNA for PCR amplification in engraftment analysis to obtain the best results. In addition to this, we strongly suggest that before interpreting a result as negative for a recipient, it is important to carefully search at a low scale for recipient-specific peaks.

Because relapsing cells are originally from bone marrow rather than from peripheral blood cells, we also suggest that it is better to use DNA of bone marrow cells to survey the engraftment condition rather than using that of peripheral blood cells. In fact in 1998, Bader presented bone marrow transplant data in which the recipient DNA of only 2 of 7 patients could be detected in bone marrow samples but not in peripheral blood.[19] In addition to this, a longer time interval in the follow-up of these patients after allogeneic PSCT is a major limitation of the sensitivity of this assay. Consequently, it is better to investigate patients at short time intervals. With frequent monitoring, the detection of mixed chimerism by chimerism analysis may alert clinicians to a high risk of relapse and allow early intervention with rapid treatment using immunosuppression or donor lymphocyte infusion therapy, or even a second PSCT. Although many methods have been used for engraftment analysis, PCR amplification of STR loci is the best choice for many clinical laboratories because it is informative, quantitative, relatively rapid, and sensitive.

In conclusion, the greatest advantage of the STR-PCR procedure over other published procedures seems to be its high rate of discrimination. It represents an easy and accurate means for quantitatively assessing relapsed DNA. Furthermore, the use of fluorescent primers enables reliable quantification without the need for additional densitometry. The method can be especially helpful in monitoring patients undergoing transplantation.

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異體周邊血液幹細胞移植後骨髓植入之定量分析

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背 景： 針對異體周邊血液幹細胞移植，目前許多植入評估的分析方法，包括限制酵素片段長度多型性 (restriction fragment length polymorphism) 分析法及多型性基因座 (polymorphic genetic loci)的聚合酵素連鎖反應 (polymerase chain reaction) 分析法，已逐漸取代傳統的評估法。爲了增進周邊血液幹細胞植入評估方法的效能，本實驗室建立一套可定量的非放射性短片段重複序列基因(quantitative short tandem repeat; quantitative STR)分析法。

方 法： 移植前先將受髓者及捐贈者的周邊白血球DNA萃取出來，再利用包含九個STR 的檢驗套組 (AmpFlSTR Profiler Plus Kit) 將受髓者及捐贈者的STR 複製擴大，經過基因定序儀 (ABI PRISM 377 DNA sequencer) 及GeneScan 2.1 分析軟體處理後，我們可找出有效的基因等位點 (informative alleles) 用來區分受贈者及捐贈者間不同處。爲了定量分析，我們必須另外以不同比例混合受贈者及捐贈者的DNA 以製作標準曲線，接者就能以此標準曲線客觀評估受贈者有多少比例的基因復發。

結 果： 本篇研究共回溯收集十位幹細胞移植病患，其中兩位受髓者在移植後追蹤的檢體發現有基因嵌合體 (chimerism) 產生，顯示可能白血病復發。一位在第三個月追蹤時，發現38.7% 的DNA 復發；另一位在第十個月追蹤發現有6.5% 的DNA 復發。

結 論： 這套方法可提供正確、敏感、且可定量及早期偵測復發的移植後追蹤分析，以提供臨床醫師更多的資訊，協助其採取適當的醫療行爲。

(長庚醫誌 2002;25:734-42)

關鍵字： 周邊血液幹細胞移植，定量短斷片重複序列基因分析法，基因嵌合體。

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