Determination of the donor:host blood cell ratio after haematological stem cell transplant by means of semiquantitative detection of short tandem repeat polymorphisms

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Summary

An improved method of quantifying the donor:host blood cell ratio after haematological stem cell transplantation (PBSCT) using a variable number of tandem repeat (VNTR) markers is presented. The post-transplant DNA is extracted from the patient's blood and amplified by semiquantitative polymerase chain reaction (PCR) without prior mock transplant and plotting of a standard curve. The amplification products are then analysed by ABI PRISM 310™ capillary electrophoresis apparatus. The resultant peak areas are correlated with the corresponding microsatellite allele by GeneScan™ software and the donor:host cell ratio is calculated. Improvements to PCR amplification conditions are essential for the outcome of the quantification since preferential amplification of alleles in the PCR process can account for the marked deviation found between the results gained by measurement of different microsatellite loci. To assess the accuracy of the method, the post-transplant blood samples of 6 patients who had undergone either myeloablative or non-myeloablative transplantation regimens were analysed retrospectively (median observation time 298 days). By analysing 3 or 4 microsatellite loci we were able to detect full engraftment or mixed chimaerism after transplant with a measurement precision of ≤4.5 (standard deviation). Sensitivity for different primers ranges from 2% to 5%. The results of the microsatellite analysis correlated well with the corresponding clinical findings. We conclude that post-transplant analysis of microsatellite loci using semiquantitative PCR without standard is suitable for clinical purposes.

Key words: semiquantitative PCR; bone marrow transplantation; microsatellite DNA; short tandem repeats

Introduction

Analysis of the donor:host blood cell ratio after allogeneic stem cell transplantation is used to assess engraftment or the degree of mixed chimaerism in the patient's peripheral blood or bone marrow. After conventional conditioning with, for instance, total body irradiation and chemotherapy, full engraftment is usually observed [1]. Mixed chimaerism has been described in T-cell depleted stem cell transplantation in particular, and is associated more frequently with relapse of the malignant disorder [2]. The introduction of low-intensity conditioning methods [3–8] with the possibility of subsequent DLI [9] and the ris-

ing numbers of allogeneic stem cell transplantations [10] makes close monitoring of the donor:patient blood cell ratio more important for clinical decision-making.

The procedure of quantitative determination of haematological stem cell engraftment using fluorescent polymerase chain reaction primers for microsatellite loci was first described by Scharf et al. [11]. The necessity of a pre-transplant standard curve obtained by DNA dilution experiments (mock transplant) is an indispensable element of the method set up by Scharf et al., since the preferential amplification of alleles during the PCR

process has been identified as an important source of analysis default. In addition, the technique demands different PCR protocols for different microsatellite loci. This method was also applied by Miflin et al. [12] to measure chimaerism after allogeneic transplantation of peripheral blood stem cells. Recently, an optimised approach for clinical routine analysis based on the method of Scharf et al. has been described by several authors [13, 14]. In these studies the problem of preferential amplification and of random variability in amplification was avoided by calculating the mean value for a kit of 7–9 STRs [14].

The aim of this study was to develop an improved approach for microsatellite analyses which minimises the preferential amplification of alleles by optimising the conditions for PCR. This renders a standard curve superfluous and makes it possible to standardise the reaction conditions for all patients and all sets of PCR primers. The changes made in the conditions for PCR in order to reduce preferential amplification are based on the following concept:

1. Preferential amplification of alleles is not predictable.
2. Sub-optimal or deteriorating PCR conditions are likely to affect the amplification of the weaker allele more and earlier than that of the stronger fragment. Consequently, preferential amplification is enhanced by sub-optimal PCR conditions.
3. Sub-optimal PCR conditions are defined by the upper limit of the logarithmic amplification of alleles (i.e. an amplification rate of <2). A fall in the amplification rate per cycle to values under 2 indicates incipient deterioration of conditions for PCR.

The precision and accuracy of the method were assessed by analysing samples of DNA mixtures with known allele ratios. Clinical samples were analysed to assess the interrelationship between laboratory test and clinical findings.

Patients and methods

Selection of patients

Patients transplanted between 1995 and 2000 were selected for the study if a prior microsatellite analysis of the respective donor had been performed and one pre-transplant as well as at least 6 post-transplant samples from the patient covering a period of more than 10 weeks were available. Moreover, at least 2 of 4 analysed microsatellite loci had to be informative for chimaerism.

The study included 6 patients treated with different conditioning regimens. Three of these patients (unique patient number (UPN) 781, 723, 673) had undergone a conventional transplantation regimen (etoposide, cyclophosphamide, TBI or busulphan, cyclophosphamide, melphalan in a paediatric protocol) whereas the other three (UPN 790, 770, 735) had been transplanted in compliance with either the Seattle (fludarabine 3×30 mg/kg + 2 Gy TBI, no T-cell depletion, mycophenolate-mofetil and cyclosporine A for immunosuppression) or the Basle low-intensity protocol (cyclophosphamide 200 mg/kg + ATG, T-cell depletion). Patients UPN 723, 673, 790 achieved complete remission (CR) after the first intervention, though this remission did not persist in patient 723. Patient 781 did not show a durable CR after the first intervention but the progressive mixed chimaerism was successfully antagonised by the administration of antithymocytic globulin (ATG) and a second infusion of donor-derived precursor cells. Patient 770 also showed a progressive mixed chimaerism after the PBPCT but was successfully converted by an infusion of donor lymphocytes (DLI). Patient 735 finally also showed a rapidly progressive mixed chimaerism. In this patient the administration of 4 DLIs did not result in improvement, but a second transplantation in compliance with the Seattle protocol proved advantageous and resulted in CR. Table 1 summarises the main features of patients whose post-transplant DNA fingerprint was analysed.

Approach of investigation

Our approach was as follows: (1) Screening of pre-transplant blood samples from both patient and donor for informative microsatellite loci. (2) Determination of the upper end of the logarithmic PCR amplification phase (i.e. an amplification rate of <2 per cycle) by assessing the influence of DNA concentration, primer concentration and cycle number on the amplification curve; definition of PCR conditions which are favourable to equal allele amplification. (3) Testing the accuracy of semiquantitative PCR by analysing samples of DNA mixtures with known allele ratio. (4) PCR amplification of informative microsatellite loci and subsequent capillary electrophoresis analysis of post-transplant samples of peripheral blood or bone marrow. (5) Correlation of the detected degree of mixed chimaerism with clinical findings.

Methods

Sample preparation. DNA was extracted from a peripheral blood or bone marrow sample. Coagulation and DNA degradation were inhibited by EDTA. The QIAamp DNA Blood Maxi Kit™ was used to purify DNA. Leucocytes contain approximately 10pg DNA per cell. Assuming an extraction rate of 95% [15] and an initial DNA amount of 10ng per PCR tube, we are able to analyse 1000–2000 leucocytes per sample, depending on the number of alleles per cell in the sample.

Determination of optimum PCR conditions. Blood samples from two healthy test persons were used in these experiments. To determine the upper end of the log-phase, the following parameters in the PCR mix were varied:
– amount of DNA initially present in the reaction tube,
– amount of primers initially present in the reaction tube,
– number of PCR cycles.

General PCR conditions: Concentration of dNTPs was 0.04 mM each, of TaqGold 0.2 U/µl. The buffer was pH 8.3, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl,

Table 1:
Clinical samples.

patient (UPN)	diagnosis	transplantation mode	DLI lymphocytes/kg	informative loci	cytognetical marker
781	CML accelerated disease	1st transplant: BMT Matched unrelated donor (MUD) of same sex, 0.99 × 10⁶ CD 34⁺/kg. 2nd transplant: PBPCT asministration of anti-thymocytic globulin and again 5.17 × 10⁶ CD 34⁺/kg same donor).		FGA, vW, THO 1	t(9;22)
723	AML FAB M2	Matched unrelated donor (MUD) of opposite sex, 5 × 10⁶ CD 34⁺/kg		FGA, vW, THO 1 Amel	+19, 12p-
673	AML FAB M5a	Haplo-identical sister, opposite sex, high stem cell dose, 11.36 × 10⁶ CD 34⁺/kg T-cell depleted.		Amel, FGA, vW, THO 1	t(8;22) (p11;q13). +11
790	AML FAB M2	Mini-transplant Seattle haplo-identical sister, 4.8 × 10⁶ CD 34⁺/kg		vW, THO 1	t(6;15) (q2;q15)
770	MDS RAEB-t	Mini-transplant Basel, opposite sex, haplo-identical sister, 10.7 × 10⁶ CD 34⁺/kg	1st: 10⁶, 2nd: 10⁶ 3rd: 10⁷, 4th: 10⁷ 5th: 10⁸, 6th: 10⁸	vW, THO 1, Amel	–
735	MDS RAEB	1st transplant: Mini-transplant Basel, haplo-identical brother, opposite sex, 11.71 × 10⁶ CD 34⁺/kg 2nd transplant: haplo-identical brother Mini-transplant Seattle, 7.27 × 10⁶ CD 34⁺/kg	1st: 10⁷, 2nd: 10⁷ 3rd: 10⁸, 4th: 10⁸	FGA, THO 1, Amel	+8

UPN = unique patient number, DLI = donor lymphocytes infusion, CML = chronic myelogenous leukaemia,
AML = acute myelogenous leukaemia, MDS = myelodysplatic syndrome, FAB = French-American-British classification of leukaemic diseases, RAEB = refractory anaemia with excess blasts, RAEB-t = refractory anaemia with excess blasts in transformation.

0.001% (w/v) gelatine. The cycling conditions were: denaturing 94 °C 45 sec, annealing 60 °C 30 sec, extension 72 °C 30 sec. Final a-addition: 30 minutes. The reaction volume in the tube was 26 μl. All runs were performed on a GeneAmp PCR System 2400™ thermal cycler, Perkin Elmer. The other parameters were varied as follows:

Amount of DNA: The following amounts of DNA were tested: 0 ng, 0.1 ng, 0.5 ng, 1 ng, 5 ng, 10 ng, 50 ng, 100 ng and 400 ng. PCR amplification was performed once with 26 and once with 28 cycles.

Amount of primers: The following amounts of primers were tested: 0 pmol, 0.1 pmol, 2.5 pmol, 5 pmol, 10 pmol, 15 pmol, 20 pmol, 25 pmol. Amplification was performed once with 10 ng DNA and once with 50 ng DNA. The experiment was repeated with 26 and 28 cycles. All other PCR conditions were as above.

Number of PCR cycles: All cycle numbers between 20 and 32 were tested once with 10 ng DNA and once with 50 ng DNA. Primer concentration was 0.6 pmol/μl. The experiment was repeated twice. All other PCR conditions were as above.

Microsatellite and sex-mismatch primers. Three fluorescence-labelled primer pairs for short tandem repeat (STR) loci and one sex-specific sequence were used as markers to monitor the donor:host blood cell ratio. Table 2 shows the primers and the labelling used to track the informative microsatellite or sex-mismatch loci. FGA and THO 1 are 5-FAM (a NHS-ester dye) labelled whereas Amel and vW are HEX (a fluorescence-amidit dye) labelled. These primer sets are commercially available from Perkin Elmer Corporation.

Capillary electrophoresis. All analyses were performed on a 310 ABI Prism Genetic Analyzer™ according to manufacturer's instructions [22]. Data were processed by GeneScan™ software. The integral of the fluorescence absorption curve (peak area) of an allele was used as the measurement unit for allele frequency in the respective original blood sample.

Clinical samples. The PCR conditions found in the optimisation experiments and used to analyse the clinical samples were chosen as described in the results section.

Calculation of donor:host cell ratio. The percentage of patient's blood cells is calculated as follows [11]:

$$\% \text{ patient's alleles} = \frac{AP_1 + AP_2}{AP_1 + AP_2 + AD_1 + AD_2}$$

where AP means peak area of a patient's allele and AD peak area of a donor's allele. The contribution of alleles shared by both patient and donor was calculated by subtraction:

$$\% \text{ patient's alleles} = \frac{AC - AD}{AC + AD}$$

where AC means common allele.

Accuracy and precision. Mixtures of DNA from two healthy control persons were analysed to determine the accuracy and precision of the semiquantitative microsatellite analysis. Samples of DNA from person A and person B were mixed in the following ratios: 9 ng A + 1 ng B, 8 ng A + 2 ng B, 7 ng A + 3 ng B, 6 ng A + 4 ng B, 5 ng

Table 2
Primer sets.

microsatellite or sex-mismatch locus	labelling	number of polymorphic alleles in the population	heterozygosity rate [16]	STR sequence	chromosome locus
FGA [17]	5-FAM	14	0.85	AAAG	4q28
Amelogenin [18, 19]	HEX	2	0.5	–	X: p22.1-p22.3 Y: p11.2
THO 1 [20]	5-FAM	5	0.81	AATG	11p15.5
vWA 31 [21]	HEX	9	0.77	AGAT	12p12-pter

FGA = alpha fibrinogen locus, THO 1 = thyrosine hydroxylase locus 1, vW = von Willebrand locus, FAM = carboxy-fluorescein, HEX = hexachloro-carboxy-fluorescein.

A + 5 ng B, 4 ng A + 6 ng B, 3 ng A + 7 ng B, 2 ng A + 8 ng B, 1 ng A + 9 ng B. The 1:1 mix of DNA A and B is of particular interest, since the equal amplification of alleles can best be judged for that ratio. Data were gained by repeating this experiment three times.

Internal control. The maximum absorbency (peak height) of an allele detected by capillary electrophoresis correlates with the degree of allele amplification and thus reflects the quality of PCR conditions. For PCR conditions selected as mentioned above, the maximum absorbency of the strongest (i.e. most efficiently amplified) allele is between 1000 and 4000 relative fluorescence units (rfu). These values were verified in the experiments for accuracy and precision (cf. above and results section). Lower maximum absorbency indicates sub-optimal PCR conditions, whereas higher absorbency means that the upper limit of logarithmic allele amplification has been exceeded. Affected values were sorted out and the analysis was repeated.

Profiler. Profiler analyses were performed following the instructions of Perkin Elmer and Applied Biosystems Corporation [13].

XY-FISH. XY-FISH was performed as a two-colour interphase FISH technique with a specific probe for Y-heterochromatin. Each data point was derived from analysis of 1000 cell nuclei.

Statistical evaluation

All experiments for identification of optimum PCR conditions were repeated and the standard deviation was calculated. For the results of accuracy and precision measurements the standard deviation and the coefficient of variation were calculated, including the data from three experiment repetitions. For clinical samples, the standard deviation, the coefficient of variation and the confidence limits γ = 95% were defined containing the data of all informative markers of a patient. In patient UPN 735 the values obtained by microsatellite and sex-mismatch detection were compared to XY-FISH and the coefficient of correlation (r) was determined. The t-test was applied to these results (t = $r \times \sqrt{(n-2)}$:$(1-r^2)$, n = number of values) and was significant for α = 5% if $|t| > t_1$-$\alpha/2$ (two-sides t-test). The p-value was given.

Results

Optimised PCR conditions. The amplification curve has a sigmoid shape with dependence on all three tested parameters, i.e., amount of DNA, amount of primers and cycle number.

Amount of DNA: For 28 cycles, doubling of DNA accounts for doubling of the fluorescence absorbency for DNA amounts below 10 ng. For 26 cycles, the limit for doubling absorbency is 50 ng DNA. The single peak height was <4000 rfu for values below the duplication limits.

Amount of primers: Increasing absorbency has been shown for primer concentrations up to 0.6 pmol/µl for 10 ng DNA and up to 0.4 pmol/µl for 50 ng DNA.

Cycle number: figure 1 shows the amplification curve obtained with different cycle numbers and two different amounts of DNA. For low cycle numbers (<28), the amplification curve corresponds to a mathematical logarithmic function. For 10 ng DNA and 0.6 pmol/µl of primers the upper limit of the logarithmic amplification curve is found after 27 cycles (cf. fig. 1). With 50 ng of DNA the limit is reached after about 25 cycles. With increased primer concentrations, improved

sensitivity was achieved at the cost of lower specificity. On the basis of those experiments, the optimum amplification conditions were defined as follows:

– Buffer: pH 8.3, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl, 0.001% (w/v) gelatine.
– DNA: 10 ng per tube
– Primer: 0.6 pmol/ml (10 pmol per tube)
– dNTPs: 1 mM each.
– Enzyme: Taq Gold, 5 units per tube.
– Reaction volume: 26 ml per sample tube.
– Cycling conditions: denaturing 94 °C 45 sec, annealing 60 °C 30 sec, extension 72 °C 30 sec. Final a-addition: 30 minutes. 27 cycles were performed.

Accuracy and precision. For all 4 primers tested with DNA dilution mixtures, the measured ratio for the 1:1 mix was between 0.9 and 1.1. The coefficient of variation ranged from 2.3% to 7.0% (see tab. 3). For the other mixtures, the coefficient of variation for the FGA primer ranged from 0.5% to 4.85%, for Amel from 2.00% to 6.41%, for THO 1 from 5.6% to 6.4% and for vW primer

Peak area dependent on cycle number
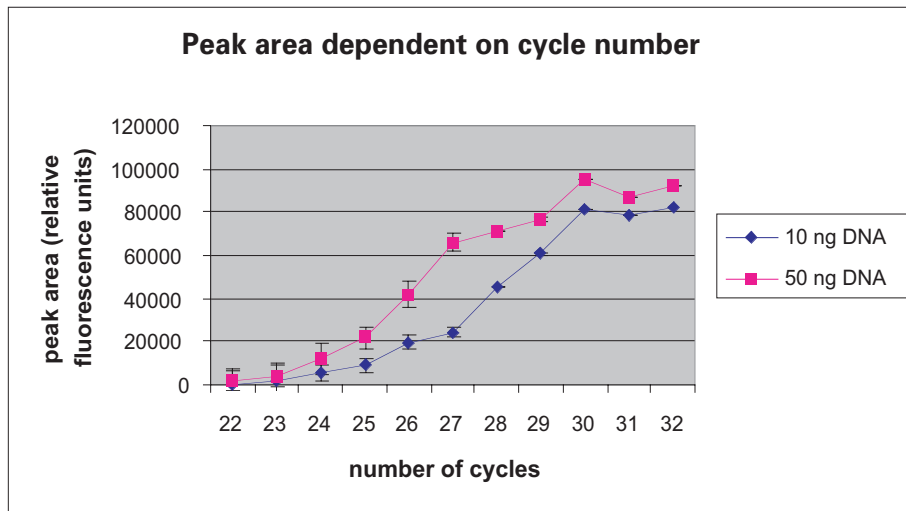
primer	measured ratio for the 1:1 mix	standard deviation	coefficient of variation
FGA	1.09	0.042	3.9%
THO 1	1.10	0.070	6.4%
vW	1.10	0.077	7.0%
Amel	0.90	0.021	2.3%

from 3.3% to 11.7%. Measurement precision is best for 5:5, 6:4 and 7:3 mixes and diminishes for 8:2 and 9:1 mixes.

Clinical samples. The post-transplant donor:host cell ratio was quantified using semi-quantitative microsatellite detection in 6 patients and the results were compared with both quantitative cytogenetic analysis and clinical findings. Samples from either peripheral blood or bone marrow were retrospectively analysed over a median observation time of 298 days.

In patient UPN 781 (fig. 2), complete chimaerism in peripheral blood was found immediately after transplantation using both the profiler method (qualitative) and the semiquantitative assessment assay. After seven weeks there is a marked increase in the patient's own alleles and within another 6 days only 25% of peripheral blood cells show the donor's fingerprint. The infusion of anti-thymocyte globulin and a second transplantation of CD34+ precursor cells results in complete cytogenetic (marker: t(9;22)) chimaerism, even though the patient remains bcr/abl-positive. In this case, the survey of the patient:donor cell ratio allows a thorough analysis of the kinetics of engraftment after the second transplantation. For the three microsatellite loci analysed the standard deviation from the mean value was <4.4%. Three STR markers were enough to achieve acceptable precision (defined as SD <4.5).

After stem cell transplantation patient UPN 723 (fig. 2) showed complete remission (cytogenetic marker: trisomy 19, 12p-). FGA and THO 1 did not feature a distinct patient's allele but the allele which was common to both patient and donor always showed the greater peak area than the donor's allele alone. We cannot decide whether this indicates a low-level mixed chimaerism or is due to preferential amplification. The analysis of the last sample showed a mixed chimaerism of more than 10%. At this time, both clinical findings and bone marrow cytology confirmed the onset of the third relapse of acute myelogenous leukaemia. The standard deviation after analysis of 4 microsatellite loci was <4.2%.

After transplantation patient UPN 673 (see fig. 2) was cytologically and cytogenetically (marker: t(8;22), trisomy 11) in complete remission. Amel, FGA, THO 1 and vW showed complete chimaerism and remained negative for patient's alleles all the time. The standard deviation for 4 microsatellite loci was <4.0%.

Patient UPN 790 (see fig. 2) shows the typical features of a Seattle mini-transplant: after two weeks, only about 10% of the blood cells showed the donor's microsatellite fingerprint. After a month, engraftment had taken place and fewer than 15% of the analysed cells featured the patient's fingerprint. The percentage of patient-derived cells detected by PCR analysis decreased further during the following month. The standard deviation found after analysis of 2 microsatellite loci was <6.7%. Acceptable precision was defined as SD <4.5. It was not possible to achieve it with only two STR markers.

Patient UPN 770 (see fig. 2) was transplanted in compliance with the Basle low-intensity protocol. Immediately after transplantation the patient showed a low-level mixed chimaerism. During the following two weeks a progressive mixed chimaerism was observed. Since the patient did not suffer from GvHD, the first DLI was started 36 days after transplant. DLI was repeated every 4 weeks and the dose was progressively increased. 20 days after the fourth DLI the percentage of patient's alleles started to decrease. After another two DLIs, the microsatellite analysis showed a formal remission of the myelodysplastic syndrome. The standard deviation for 3 analysed microsatellite loci is <4.3%.

Patient UPN 735 (see fig. 2) never achieved

Figure 2

Donor: host blood cell ratio and correlation with clinical findings in 6 patients after haematological stem cell transplantation.
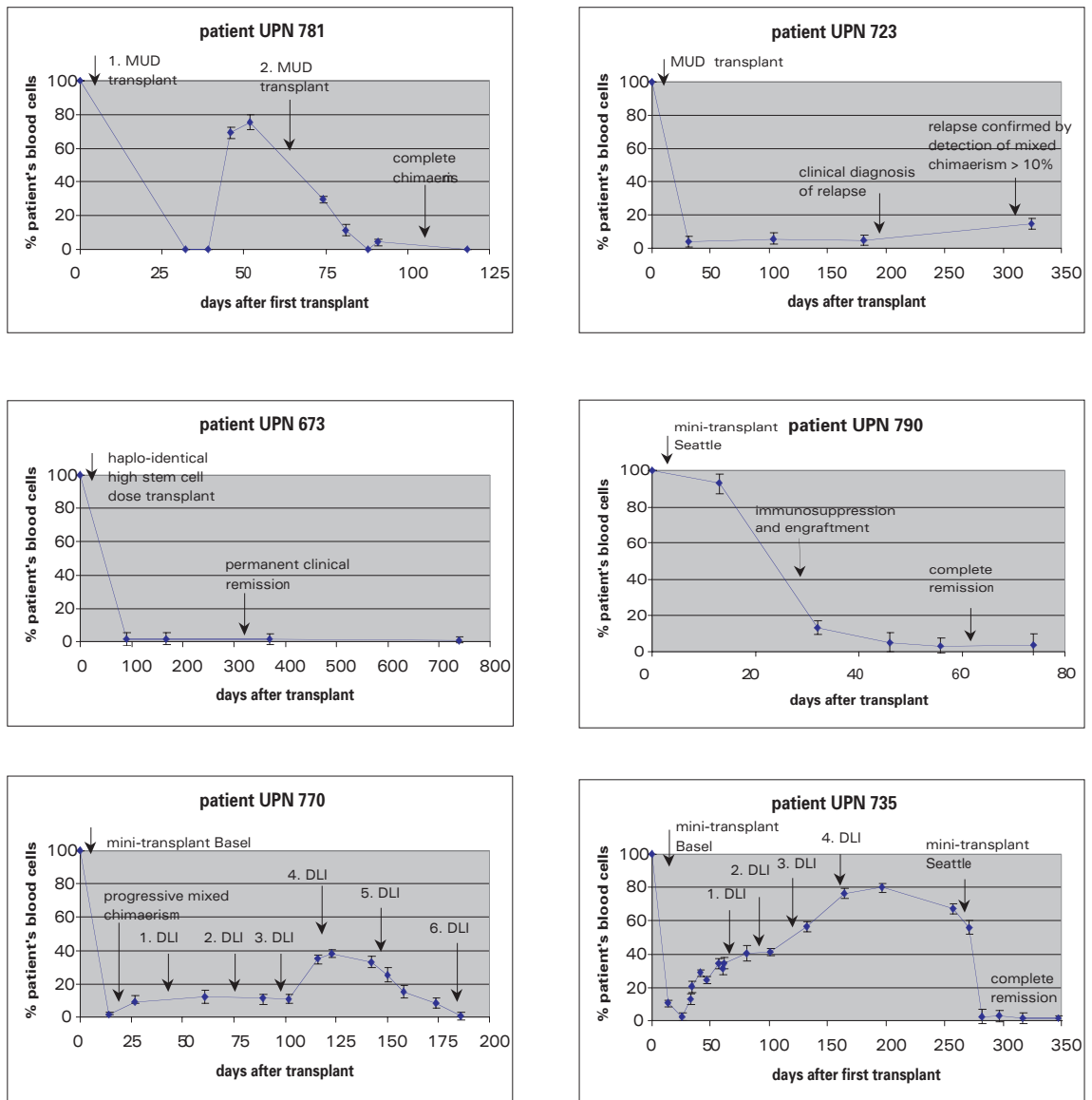
complete remission after the first transplantation. The FISH analysis (marker: trisomy 8) revealed 3.5% of trisomy 8⁺ cells. The fingerprint shows a progressive chimaerism which quickly reaches the 30% mark. Two months after transplant, 11% of the cells analysed by FISH showed trisomy 8. Since the percentage of leukaemic cells was quite considerable, it was decided to start DLI with an infusion of 10⁷ lymphocytes/kg. The amount of cells showing the patient's fingerprint went on increasing. It is remarkable, however, that analysis of a blood sample 257 days after transplantation

showed a decrease in patient's alleles before the second conditioning was initiated. Two weeks after the second transplantation, cytogenetic analysis indicated engraftment and analysis of the DNA fingerprint revealed a diminishing percentage of patient's alleles. Three months after the second transplantation, complete remission was achieved. The standard deviation for 3 analysed microsatellite loci was <4.5%. The coefficient of correlation between XY-FISH and semiquantitative microsatellite detection was 0.97, t = 11.9 (significant for t >10.2), p <0.04 (fig. 2).

Discussion

The aim of this study was to improve a blood analysis method which is able to determine the donor:host blood cell ratio in patients who have undergone blood stem cell transplantation. The method is based on accurate measurement of microsatellite alleles by means of PCR and subsequent analysis of the amplification products by capillary electrophoresis. The crucial step in this process is the reduction of preferential amplification of certain alleles during the PCR reaction. Avoidance of preferential amplification renders a pre-transplant standard curve superfluous and

allows standardisation of the corresponding analysis protocols. Optimum PCR conditions were achieved by reducing the cycle number to 27, the amount of DNA to 10ng and the amount of primers to 10pmol per tube. It is not yet clear whether increased amounts of DNA, which may account for a higher sensitivity, can be used without loss of accuracy and precision.

We analysed 70 samples of either peripheral blood or bone marrow. The donor:host cell ratio was determined in every sample. To achieve statistically satisfying results (defined as standard deviation <5%) it was necessary to include at least 3 different microsatellite primers in the analysis. The standard deviation of the mean value was always less than 4.5% if 3 or 4 microsatellite loci had been analysed and the coefficient of variation was between 0.5% and 11.7%. The confidence limits (γ = 95%) never exceeded the value of ± 4.8%. Comparison of the results obtained by analysis of the DNA fingerprint with the values indicated by quantitative FISH analysis of XY-chromosomes showed a good correlation. For different alleles, the coefficients of correlation were between 0.96 and 0.99. The t-test value was 11.9 (significant for t >10.2 if α = 5%) and the p-value <0.04. Engraftment or engraftment failure were correctly reflected by the fingerprint, and the effect of therapeutic measures on the patient could be quantified. The method of quantitative determination of bone marrow transplant engraftment described by Scharf et al. achieved a precision of 3.5–8% (coefficient of variation) if a standard curve had been plotted [11]. The method published by Tiede et al. showed a precision of 0.7–9.6% (coefficient of variation) if a median value of 7 [13] or 8 [14] STR markers had been included. They found that the coefficient of correlation between standard FISH and STR-PCR was 0.97 [13]. We conclude that it is possible to achieve a similar precision to that described in the methods of Scharf et al. and Thiede et al. including only three STR markers for analysis and omitting a standard curve, if adequate improvements have been made in the PCR reaction conditions.

Further development is needed to establish the detection of mixed chimaerism by semiquantitative microsatellite analysis as a diagnostic and prognostic tool. First, it would be useful to analyse different leukocyte subsets, e.g. the T cell fraction or the CD 34⁺/CD 19⁺ fraction, since the chimaerism of these subtypes is thought to be a more important prognostic factor for successful engraftment and residual disease eradication. In addition, this may result in a two-log increase of sensitivity in the identification of potentially malignant donor-derived cells [23]. Second, attempts could be made to detect polymorphic sites in mitochondria and thereby reduce the number of cells needed for an analysis by the factor 100. Finally, new technologies such as the Taqman analyser may allow simpler and more accurate analysis of cell ratios by quantifying polymorphic single-nucleotide loci.

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