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Chimaerism

Analysis of haematopoietic chimaerism by quantitative real-time polymerase chain reaction

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Summary:

Allogeneic bone marrow transplantation (BMT) with marrow ablative conditioning is the treatment of choice for haematopoietic malignancies. The use of nonmyeloablative stem cell transplants has allowed the treatment of patients previously ineligible for BMT because of age or other disease. These reduced conditioning regimes allow the persistence initially of some recipient cells in the blood and bone marrow (haematopoietic chimaerism). Monitoring of the relative proportion of donor and recipient cells is required to assess the success of the procedure, to predict subsequent rejection or impending relapse and to guide the use of donor lymphocyte infusions. We present a quantitative real-time PCR approach for the measurement of haematopoietic chimaerism using the TaqMan™. This approach exploits the presence of single-nucleotide polymorphisms (SNPs) to distinguish cells of patient or donor origin. We have designed and validated a panel of seven allele-specific probes to quantify the contribution of patient and donor cells in the haematopoietic population from 12 patient and donor pairs. We have compared the performance of this approach with an existing method and proved it to be superior in both accuracy and sensitivity. The use of more sensitive and accurate techniques permits earlier intervention for improved clinical outcome.
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Allogeneic bone marrow transplantation (BMT) is the treatment of choice for a wide range of haematopoietic disorders. Traditionally, the patient has been subjected to high-dose conditioning regimes prior to transplantation. The intense nature of this treatment has rendered a large number of patients unsuitable for treatment by virtue of age (graft-versus-host disease (GVHD) is more common in patients over 55 years old) or underlying organ damage. Mortality in the first 100 days post transplant depends on a number of factors. Overall mortality is around 10–15%, but in some situations can be as high as 30–50%.[1] The finding that the remissions were not solely due to the cytotoxic effects of the conditioning but were also a product of a graft-versus-leukaemia (CVL) effect[2] has allowed the use of less-intensive procedures such as nonmyeloablative stem cell transplant[3,4] or reduced conditioning allograft. However, disease relapse, graft rejection and GVHD remain a considerable problem. The persistence or reappearance of recipient cells (mixed chimaerism; MC) following nonmyeloablative SCT has been associated with impending relapse,[5,6] increased risk of GVHD[7] and graft failure.[8] Conversely, persistent full donor chimaerism is consistent with disease free-survival.[9] Khan *et al*[10] have recently highlighted the importance of chimaerism monitoring for prediction of such adverse events.

Methods of determining the persistence of patient cells in the population have been recently reviewed by Thiede[11] and include RFLP analysis,[12] VNTR analysis,[13] sex-specific mismatch[14] and microsatellite analysis.[15,16] The sensitivities of these techniques vary greatly. Minisatellite and microsatellite analysis are the most widely used, with accuracy and sensitivity estimated between 0.1 and 5% patient cells.[9,13,15,17–19] However, the accuracy of this approach is dependent on the choice of marker, since measurements may be complicated by the presence of 'stutter' peaks arising from slippage of the DNA polymerase during PCR. For this reason, tetranucleotide repeats are preferred to dinucleotide markers and the discriminant allele should not be the same size as any stutter peak.

An alternative source of genetic variation between patients and donors are single-nucleotide polymorphisms (SNPs). The majority of SNPs are biallelic (they exist as two forms differing at a single nucleotide position) and they are present at approximately 1 SNP per 1000 bases throughout the human genome.[20] Identification of SNP differences between patient and donor can be used to identify the origin of cells within a population. SNP differences between individuals have recently been used in microarray-based minisequencing[21] and pyrosequencing[22]

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approaches to determine chimaerism status. In these studies, data produced from SNP analysis correlated well with that obtained from examination of microsatellites.[21]

The recent application of real-time PCR has allowed accurate quantitation of nucleic acids.[23] Real-time PCR using the ABI TaqMan™ Prism 7000 instrument relies upon the detection of PCR products during the elongation phase of amplification by the binding of allele-specific probes to target sequences. The probes are labelled 5′ with a fluorochrome and 3′ with a nonfluorescent quencher molecule, and are quiescent when in free solution. Where probes are perfectly bound, cleavage of the fluorochrome from the quencher during PCR produces a fluorescent signal. The level of fluorescence is a direct measurement of the amount of template. The starting concentrations of any given fragment can be determined as a function of the point at which the fluorescent signal becomes visible above the background (crossing point; C^t). This SNP-typing approach can be used to detect haematopoietic chimaerism.[11,24–26]

In the current study, we exploit the presence of SNPs to identify differences between patient and donor. We present a panel of allele-specific TaqMan™ probes to assess chimaerism in post transplant patient samples. We compare results achieved with the microsatellite approach with those achieved from real-time PCR analysis from artificial chimaeric mixtures of known composition. This assay permits the rapid, accurate and sensitive quantitation of haematopoietic chimaerism from both blood and bone marrow in patients following BMT.

Patients, materials and methods

Patients

Six bone marrow samples and 33 peripheral blood samples were received from patients referred for nonmyeloablative SCT from the Royal Devon and Exeter NHS trust and Plymouth Hospitals NHS trust as part of their routine post transplant monitoring. We analysed 12 patient:donor pairs. Both sibling ($n = 9$) and unrelated donors were represented ($n = 3$). Patient diagnoses included AML ($n = 6$), CML ($n = 3$), NHL ($n = 1$) HL ($n = 1$) and MCL ($n = 1$).

Sample preparation

DNA was extracted from peripheral blood and bone marrow aspirates by previously described techniques.[27] The concentration of DNA was measured by UV spectrophotometry.

Microsatellite analysis

Samples were analysed for MC by the use of a multiplex PCR containing primers for seven microsatellite loci and one set of primers for the amelogenin gene that distinguishes X and Y sequences (Table 1a). Briefly, PCR reactions contained 1.5 mM $MgCl_2$, 0.8 μM dNTPs (ABgene, Epsom, UK), 0.5 U *Taq* polymerase (ABI, Warrington, UK) and 40 ng DNA in a total volume of 25 μl. Primer concentrations are given in Table 1a. PCR cycling conditions were a hot start of 95°C for 10 min, followed by 12 cycles of 94°C – 30 s, 55°C – 30 s and 72°C – 1 min and 13 cycles of 89°C – 30 s, 55°C – 30 s and 72°C – 1 min. PCR products were analysed on an ABI 377 using Genotyper™ software (ABI, Warrington, UK).

Informative markers were identified and standard curves were constructed of 0, 1, 5, 10, 25, 50, 75 and 100% patient DNA diluted in donor DNA for each informative marker. PCRs were halted after 25 cycles to ensure that the reaction was in the exponential phase and the results would be quantitative. The percentage of patient cells was calculated as a function of the ratio of patient and donor peak areas for each dilution (SP/SD). The percentage of patient DNA for the test samples could then be determined from the graph (Figure 1).

Real-time assay development

SNPs were chosen from the dbSNP database[28] on the basis of biallelic status and high levels of heterozygosity in the population. We calculated that a marker with a frequency of 0.5 for each allele would be most informative. The TaqMan™ system relies upon competition for binding to target of a pair of probes, one specific for each allele.[23] The probes are labelled 5′ with either 6-FAM or VIC. The incorporation of a 3′ minor-groove-binding protein into the probe raises the melting temperature of the probe so that shorter and therefore more specific probes can be used. Specific TaqMan™ MGB assays were designed using the Assays-by-design™ service from Applied Biosystems (TSC0955234, rs3918344 and rs338773) or by Primer Express™ software supplied with the instrument (TSC0005295, TSC0541887, Amelogenin and T515[29]). Primer and probe sequences are given in Table 1b. The amelogenin assay necessitated the use of two alternate reverse primers in the reaction, as the X chromosome PCR product was too large for the TaqMan™, which has an ideal amplicon size of less than 150 bp. Each probe pair was validated for accuracy by the construction of standard curves from serial dilutions of 8 ng to 250 pg. Each point on the curve was set up in triplicate to allow calculation of standard error. Probes were then validated for sensitivity by mixing experiments involving dilution of patient DNA in donor DNA. Specificity was determined by the use of 100% patient and 100% donor DNAs. Some crosstalk between dyes was expected but this was accounted for by colour compensation functions in the quantitation software.

Identification of informative markers for each patient

Patient:donor pairs were tested with the TaqMan™ probe panel to identify informative markers. Markers were determined to be informative if either the patient or the donor possessed a unique allele or alleles (ie heterozygosity or homozygosity for disparate alleles). PCRs were carried out in 25 μl reactions containing 12.5 μl TaqMan™ master mix (ABI, Foster City, USA), 200 nM each probe, 250 nM each primer and 50 ng DNA. Cycling conditions were: one cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min.

Table 1 Microsatellite panel (a) and SNP[a] panel (b)

(a) Microsatellite	*Primer*	*Primer concentration (μM)*	*Label*
D20S107-F	5′ CTA CAT GAT GCC TCT TGG GA 3′	1	FAM
D20S107-R	5′ GGT TTC AGA CAA TGG CAA ATT CCT 3′	1	
D20S851-F	5′ CAA CTT CAA GTT ATG TGT GGC ACA A 3′	0.4	HEX
D20S851-R	5′ GCC AGA CTC TGA CAC CTT T 3′	0.4	
D20S103-F	5′ CAT AGA GGG ACA AGA CAC AGT 3′	0.3	TET
D20S103-R	5′ GCC ATG ATG TTT GGT TAA TCA CA 3′	0.3	
D20S477-F	5′ CAG GAA TAA CTC AGG GTG TCT TG 3′	1	TET
D20S477-R	5′ GAG ACG GCT CCC TTA ACA CA 3′	1	
D20S482-F	5′ CAG CCT CCA TAA CCA CAT GAA 3′	0.3	TET
D20S482-R	5′ GAA CCT AAA ACT CTA AGG AAG CG 3′	0.3	
D20S171-F	5′ GGT GAG GAC CCT GAG G 3′	0.3	HEX
D20S171-R	5′ GAC CAA GCC ATG TAA CCT G 3′	0.3	
D20S481-F	5′ TGG GTT ATG AGT GCA CAC AG 3′	0.6	HEX
D20S481-R	5′ GAC AGC AAA AAG ACA CAC AGC 3′	0.6	
D2S206-F	5′ TTA AAA ATT AAG TAG GCT TTT GGT T 3′	0.5	HEX
D2S206-R	5′ GTC CTC ATG TGT TTA TGC TGT 3′	0.5	
Amelogenin-F	5′ CTG ATG GTT GGC CTC AAT CCT 3′	0.3	FAM
Amelogenin-R	5′ ATG AGG AAA CCA GGG TTC CA 3′	0.3	

(b) SNP	*Function*	*Modifications*	*Sequence*
TSC0955234F	Primer	None	5′ cta cac tct aaa aac aaa tgg acc taa cag a 3′
TSC0955234R	Primer	None	5′ ctg tcg tgt gga cta tct tga aga g 3′
TSC0955234(A)	Probe	5′ VIC, 3′ MGB	5′ gc tga tta gga taa tgt at 3′
TSC0955234(C)	Probe	5′ 6-FAM, 3′ MGB	5′ ctg att agg aga atg tat 3′
TSC0005295F	Primer	None	5′ cct tgc ttc aaa tgc caa aag 3′
TSC0005295R	Primer	None	5′ ggc aca cct tag gag gaa tcc 3′
TSC0005295(A)	Probe	5′ VIC, 3′ MGB	5′ cct cag **c**at gcc tg 5′
TSC0005295(C)	Probe	5′ 6-FAM, 3′ MGB	5′ cct cag **a**at gcc tg 3′
TSC0541887F	Primer	None	5′ tga agc cta cag taa aaa ccc taa tg 3′
TSC0541887R	Primer	None	5′ gat gcc tag ggc aaa gta ttg g 3′
TSC0541887(T)	Probe	5′ VIC, 3′ MGB	5′ aga ggg cat g**t**a acc 3′
TSC0541887(G)	Probe	5′ 6-FAM, 3′ MGB	5′ aga ggg cat g**g**a ac 3′
rs3918344F	Primer	None	5′ gac agg gag tca tca ata gta aca tga a 3′
rs3918344R	Primer	None	5′ ggt atg tgc agt tgg gtg cta 3′
rs3918344(C)	Probe	5′ VIC, 3′ MGB	5′ tca gga gac a**c**a ttt g 3′
rs3918344(T)	Probe	5′ 6-FAM, 3′ MGB	5′ cag gag aca **t**at ttg
rs338773F	Primer	None	5′ agc agt gcc taa ggc ata gaa aat 3′
rs338773R	Primer	None	5′ caa cga tat ggt gta tct gag tca tgt 3′
rs338773(A)	Probe	5′ VIC, 3′ MGB	5′ caa tat aaa atg tgt c**a**t aag aaa 3′
rs338773(G)	Probe	5′ 6-FAM, 3′ MGB	5′ aaa tgt gtc **g**ta aga aa 3′
T515F2	Primer	None	5′ ctg cgg cca gcc ctc ta 3′
T515R	Primer	None	5′ gtg gtg tcg gtg atg agc ata gt 3′
T515A	Probe	5′ VIC, 3′ MGB	5′ aca ccc aca c**a**g gc 3′
T515G	Probe	5′ 6-FAM, 3′ MGB	5′ cac cca cac **g**gg c 3′
AmelF	Primer	None	5′ ctg atg gtt ggc ctc aag c 3′
AmelR	Primer	None	5′ atg aga aaa cca ggg ttc ca 3′
AmelR2	Primer	None	5′ cta atc ctt aca ttt tac tag ctg ggt tag 3′
AmelY	Probe	5′ VIC, 3′ MGB	5′ **agt aaa ctc tga cca gct** 3′
AmelX	Probe	5′ 6-FAM, 3′ MGB	5′ **tca act ctg act gac cag** 3′

[a]Probe and primer sequences are given. The site of the SNP is marked in bold type.

Real time quantitative PCR

Real-time quantitative PCRs were carried out by standard TaqMan™ technology using the ABI PRISM 7000 platform. The signal produced by binding of the allele-specific probes was measured. The probes were labelled with two fluorochromes with different emission spectra to allow simultaneous amplification in a single tube. The fluorescent signal from each allele is proportional to the amount of product. The ABI PRISM 7000 calculates the point at which the fluorescence signal rises above the background; the crossing point (C^t). This is directly proportional to and thus a direct measure of the starting template.

We aimed to calculate the relative frequency of patient and donor DNA by identifying the relative ratio of alleles at the informative SNP. Where evidence of MC was found, standard curves were set up in triplicate for informative markers. Following PCR amplification, the quantity of each allele was determined from the standard curve derived by the TaqMan™ software. The percentage of heterozygous (H) and homozygous (h) DNA was calculated by the equation below, where R = ratio of unique allele to common allele normalised to a heterozygous control, to account for slight differences in probing efficiency. The equation was derived from the proportion of unique alleles as a percentage of the total alleles, and the fact that the

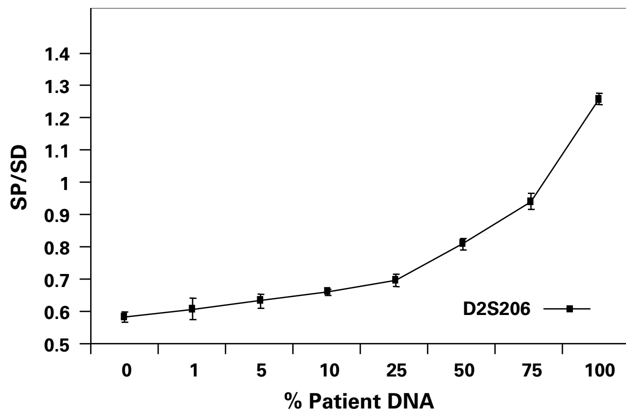

Figure 1 Standard curve analysis of microsatellite marker D2S206. Triplicate standard curves for marker D2S206 were constructed of 0, 1, 5, 10, 25, 50, 75 and 100% patient DNA diluted in donor DNA. Error bars represent 2 standard deviations. The ratio of patient alleles to donor alleles (SP/SD) is plotted on the *Y*-axis and the input recipient DNA is plotted on the *X*-axis.

unique allele must be paired with a common allele in an individual heterozygous at that locus.

$$H = 2\left(\frac{100}{100 + (100R)}(100R)\right)$$

$$h = 100 - H$$

In a situation where patient (*HH*) and donor (*hh*) are homozygous for disparate alleles at a locus, the percentage of patient DNA is given by the equation given below.

$$HH = \left(\frac{100}{100 + (100R)}(100R)\right)$$

Comparison of microsatellite and real-time PCR approaches

Artificial chimaeric mixtures were created using two DNA samples that were homozygous for different alleles of two SNPs and heterozygous for two microsatellite markers. The microsatellites included the tetranucleotide marker D20S481 and the dinucleotide marker D20S107. The informative 'patient' allele of D20S107 did not coincide with any stutter peak from the 'donor' alleles. The mixtures ranged from 0 to 100% of DNA sample 1 ('patient') relative to DNA sample 2 ('donor') using a total of 40 ng DNA. Triplicate PCRs were set up from the same chimaeric DNA samples by microsatellite and real-time methods. Analysis was carried out as described above.

Results

Assay design

We identified seven biallelic SNPs with a heterozygosity of approximately 50% in at least two ethnic groups. We designed assays only to SNPs for which genotyping data were available to avoid the selection of variants that might be present only *in silico* (ie variants present only in the database that probably represent sequence artefacts rather than true SNPs). If both homozygotes and heterozygotes are taken to be informative, an SNP with an allele frequency of 0.5 will be informative for 62.5% of patient: donor pairs.[24] Therefore, statistically, four loci should provide an informative marker in any situation,[24] although additional markers may be required for some patients. All assays designed from these SNPs produced reliable and specific amplification.

Validation of assay

The accuracy of each SNP assay was determined by serial dilution of heterozygous DNA. In addition, the specificity of probing was monitored by the inclusion of 100% patient and 100% donor DNA into the series. The standard curves produced were seen to be linear over all ranges with small variations in the C^t values achieved for each dilution. All seven TaqMan™ assays yielded consistent and efficient amplification with no major variations in PCR or probing efficiency between alleles as shown by standard curve analysis. All assays proved sensitive to 250 pg of DNA (Figure 2a). An optimally efficient PCR reaction yields a slope of −3.3 on the standard curve, with *Y* intercept values (theoretical value for detection of one template molecule) between 36.1 and 45.99 cycles (average 41.26, s.d. 3.5). Efficiency of amplification for our assays was −3.5 (mean value; range −3.23 to −3.78). The correlation coefficient (r^2 value) of standard curves was 0.98 overall, with a range of 0.97–0.98.

We determined the sensitivity of each SNP assay by artificial chimaeric mixtures (serial dilutions of patient DNA in donor DNA). Mixing experiments indicated that the assays are capable of accurately detecting 1% patient DNA (Figure 2b).

Qualitative and quantitative analysis of unknown samples

To assess the performance of our assay in post transplant monitoring of haematopoietic chimaerism, we monitored 12 recipient:donor pairs over a period of 3 years (Table 2). Initially, all 24 individuals were genotyped with each marker in the panel and also with a panel of microsatellite markers (Table 1b). Figure 2c shows an example of genotyping data obtained with the SNP assay. All patient: donor pairs proved informative for at least one SNP and 92% of pairs were informative for two or more SNPs. In all, 92% of patient donor pairs were informative using this panel of seven microsatellite multiplex reaction and only one patient:donor pair (pair 9) required the use of an additional microsatellite. Sex mismatch was noted in 25% of patient:donor pairs. Post transplant samples for each recipient were analysed for evidence of MC using both real-time and microsatellite analysis. In total, 10/12 (83%) patients showed a full donor profile (FDP) at all times by both real-time and microsatellite analysis. Samples showing evidence of MC were then quantified by real-time PCR and microsatellite analysis. Mean error in quantitation of MC for the real-time PCR assay was 4.9% (range 2.0–11%).

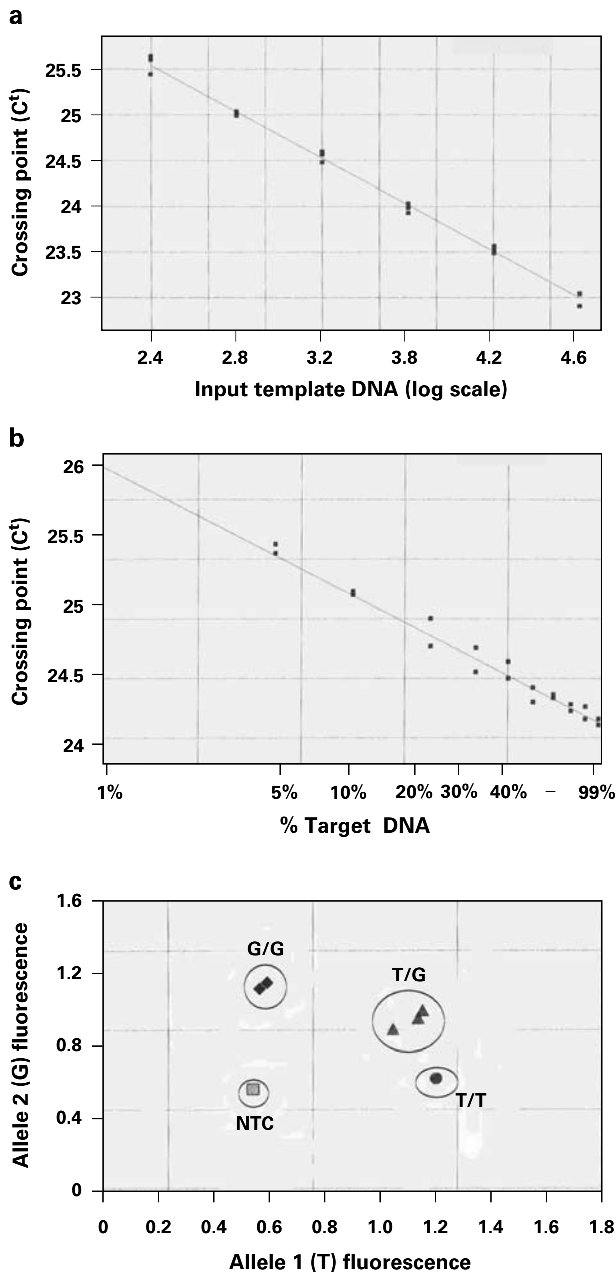

Figure 2 (**a**) Standard curve for serial dilution of DNA heterozygous for TSC0541887. Standard curves were constructed from triplicate serial dilutions of heterozygous DNA from 8 ng to 250 pg. The crossing point (C^t) is plotted on the *Y*-axis and the log of the input template DNA is plotted on the *X*-axis. The curve is seen to be completely linear, with a slope of −3.23 and a correlation coefficient of 0.98. (**b**) Standard curve produced by mixing of DNA samples homozygous for different alleles of TSC0541887. Standard curves were constructed by mixing two DNA samples homozygous for disparate SNP alleles in known proportions from 1 to 100%. The percentage of the target allele is plotted on the *X*-axis against the crossing point C^t on the *Y*-axis. (**c**) Genotyping data for SNP marker TSC0541887. The fluorescence from allele 1 (T) is plotted on the *X*-axis against the fluorescence of allele 2 (G) on the *Y*-axis. Homozygous G/G samples are given by diamonds, heterozygous G/T samples by triangles and homozygous samples T/T by circles. The no template control is designated by NTC. The three genotypes can clearly be seen separate from the no template control.

Comparison of microsatellite and real-time methods of quantitation

The composition of a series of artificial chimaeric mixtures was determined by microsatellite and real-time approaches. The results are given in Figure 3a and b. There was some intermarker variation in the quantitation achieved by both methods. The correlation coefficient (r^2) between the calculated composition and the known composition was 0.994–0.998 for the real-time technique and 0.991–0.992 for the microsatellites, respectively. Comparison of intermarker variability revealed that the real-time method achieved reproducible and accurate results over all chimaeric ranges with r^2 values of = 0.99, 0.99 and 0.99 for the total range, $<20\%$ target and $>80\%$ target, respectively. The microsatellite method performed well over the mid-range of target concentrations ($r^2 = 0.98$) but proved less reliable at the extremes of the template range (target at <20 and $>80\%$ of the mixture) when r^2 was 0.80 and 0.96, respectively. Microsatellite D20S481 failed to detect any patient DNA below a threshold of 20% (see Figure 3a).

Clinical results

We present two clinical situations in which the utility of our new approach is demonstrated.

Case 1. Patient 12 is a 64-year-old man with CML. He underwent a nonmyeloablative SCT with an HLA-matched sibling donor. We analysed nine sequential blood samples. The patient showed a partial response accompanied by evidence of MC, which increased until week 14 post transplant. This correlated with the re-emergence of his disease. The patient was then treated with STI-571, which targets the t(9:22) translocation fusion product. He also received donor lymphocyte infusion (DLI) at week 14 after which a progressive decrease in mixed chimaerism was noted until a full donor profile was noted at week 19. He is currently asymptomatic and in full molecular and cytological remission as judged by absence of the t(9:22) translocation chromosome by fluorescence *in situ* hybridization (FISH) or reverse transcriptase (RT) PCR.

Case 2. Patient 8 is a 37-year-old man diagnosed with MCL. He underwent nonmyeloablative SCT treatment with an HLA-matched sibling donor. This patient also showed MC in both peripheral blood and bone marrow 10 weeks after transplant that persisted until week 22. Mixed chimaerism was more marked in the bone marrow (57% patient cells) than the peripheral blood (37% patient cells) at week 10. He received a DLI at this point and showed a full donor profile in samples taken at week 42 and week 70 post transplant.

Discussion

We have developed a novel quantitative real-time PCR technique to rapidly and accurately detect and monitor the

Table 2 Characterisation of patient samples

Patient	*Disease*	*Relationship to donor*	*Number of informative microsatellites*	*Number of informative SNPs*	*Sex mismatch*	*Chimaerism status*
1	HL	S	2	2	Y	FDP
2	AML	S	2	1	N	FDP
3	CML	S	2	2	N	FDP
4	AML	S	3	4	Y	FDP
5	AML	S	2	3	N	FDP
6	AML	U	3	3	Y	FDP
7	AML	U	3	5	N	FDP
8	MCL	S	1	2	N	MC
9	NHL	S	4	1	N	FDP
10	AML	U	2	4	N	FDP
11	CML	S	3	2	N	FDP
12	CML	S	2	2	N	MC

HL = Hodgkin's lymphoma; AML = acute myeloid leukaemia; CML = chronic myeloid leukaemia; MCL = mantle cell lymphoma; NHL = non-hodgkin's lymphoma.

incidence of MC in patients following nonmyeloablative SCT BMT. An increasing degree of MC has been correlated with impending relapse,[5,6] GVHD[7] and graft failure[8] in a number of studies. Analysis of lineage-specific samples may prove especially useful since mixed chimaerism is often more prevalent in T-cell-depleted samples.[6,16,30] Any method of assessing haematopoietic chimaerism must be fast, sensitive, accurate and applicable to all patients regardless of disease or relationship to donor. Methods such as RFLP analysis,[12] VNTR analysis,[13] sex-specific mismatch[14] and microsatellite analysis[15,16] have been used to monitor MC but these methods lack the accuracy of quantitation and speed of real-time PCR.

SNPs are particularly useful for chimaerism analysis because the differentiation between patient and donor is based on interindividual variation and does not rely on disease-specific markers. The high information content of the SNP panel means that any two individuals can in theory be distinguished. For example, Hochberg *et al*[22] were able to distinguish at least one informative locus in 55 patients with HLA-identical donors with a panel of 14 SNPs.[22] Other SNP-based approaches using microarray-based minisequencing[21] or pyrosequencing[22] techniques have proved superior to microsatellite methods on the basis of turnaround time or accuracy, but are less sensitive than real-time PCR methods. For example, a pyrosequencing technique had a reported detection limit of 5%.[22]

Real-time PCR has previously been used to monitor the CML specific t(9:22) fusion product.[31,32] Other studies have used the polymorphic cytochrome P450 *CYP2C9* gene and used end point measurements for quantitation of mixed chimaerism.[24] It is now well recognised that crossing points give a more accurate determination of starting template than end point measurements.[23] Subsequent studies produced a large panel of SNP-based TaqMan™ probes but discriminated patient and donor on the basis of allele-specific amplification detected with a common probe.[25,26] The use of allele-specific oligonucleotides in both these studies permits allele discrimination but raises the caveat that alleles may not be amplified equally due to differences in the efficiency of the PCR. Using this approach Maas *et al*[26] found high standard errors in cases where the contribution of patient cells to the DNA population was elevated, necessitating the use of two separate standard curves to accurately determine the degree of MC.[26] The use of common primers and an allele-specific probe system represents an improvement on current methods since the detection and quantification of patient and donor DNA is independent of PCR efficiency. Our approach allows the quantitation of one allele relative to the other in the same tube from a single standard curve. The use of a single common pair of primers and a single tube approach effectively controls for PCR inhibitors, differences in template input and allows more accurate quantitation.

We have performed a comparison between the use of microsatellites and SNPs to distinguish DNA from two origins for the purposes of chimaerism analysis. The amount of patient cells present in a series of artificial chimaeras was determined by both approaches. Both methods performed well over the middle of the measured target range, but the microsatellite approach was less accurate at either low or high target concentrations. Although general estimates of the linear range of microsatellite markers cannot be drawn from studies of single microsatellites, intermarker variation is apparent (see Figure 3a). The linear range for the trinucleotide repeat microsatellite D2S206 as tested here is only between approximately 25 and 75% (Figure 1). In contrast, the standard curve produced by our novel TaqMan™ technique is reliably linear over all ranges. This has clinical importance in that patients who may be about to relapse (with low but increasing amounts of MC) may not be accurately identified by microsatellite methods. We believe that due to its superior sensitivity, real-time PCR is the method of choice for chimaerism-based minimal residual disease monitoring. The application of the new technique in lineage-specific studies may increase the sensitivity of the approach further.

To conclude, we report a new method for the evaluation of haematopoietic chimaerism following BMT. This method is fast, accurate and sensitive. Improved quantitation of the persistance or re-emergence of patient cells in the post transplant blood or bone marrow of patients suffering from haematological disorders has the potential

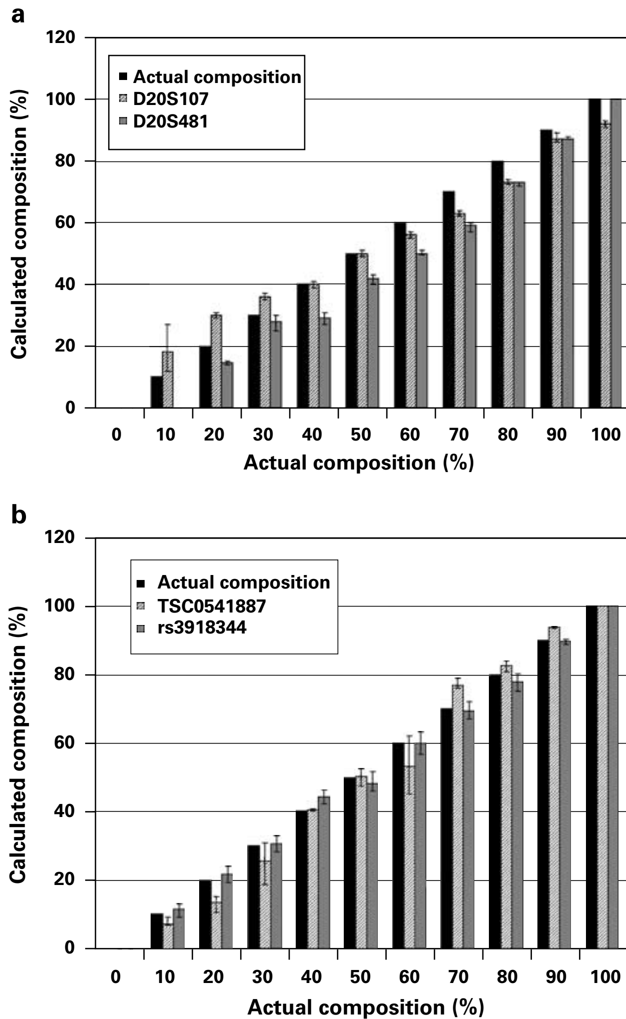

Figure 3 (**a**) Microsatellite quantification of chimaerism. The known composition of the mixture is plotted on the *X*-axis against the calculated composition on the *Y*-axis. Black boxes represent the absolute composition, hatched boxes represent marker D20S107 and grey boxes represent marker D20S481. Quantifications are based on triplicate measurements. The error bars represent the upper and lower limits of the measurement. (**b**) Real-time PCR quantification of chimaerism. The known composition of the mixture is plotted on the *X*-axis against the calculated composition on the *Y*-axis. Black boxes represent the absolute composition; hatched boxes represent measurements from marker TSC0541887 and grey boxes represent measurements from marker rs3918344. Quantifications are based on triplicate measurements. The error bars represent the upper and lower limits of the measurement.

to allow early detection of emergent leukaemic populations and thus improve clinical outcome.

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