Use of Single Nucleotide Polymorphisms (SNP) and Real-Time Polymerase Chain Reaction for Bone Marrow Engraftment Analysis

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Allogeneic bone marrow transplant engraftment assays use polymorphisms in the human genome to determine the relative percentages of donor and recipient cells present in the recipient. We describe a novel posttransplant assay approach using single nucleotide polymorphisms (SNPs), the most common type of polymorphism in humans. Using samples of defined genotype, we used real-time polymerase chain reaction (PCR) and allele-specific fluorescent TaqMan probes to assay a SNP of the cytochrome P₄₅₀ CYP2C9 gene. Standard curves of chimeric mixes showed a linear relationship between the ratio of two alleles and the ratio of their respective fluorophore emission, except for mixes with a low percentage (<5%) of the less common allele. We validated the SNP real-time PCR assay by comparing it to Southern hybridization analysis, analyzing DNA mixes in a blinded fashion with both methods. The correlation between the two methods was high. We have produced a statistical model that varies allele frequency to predict how many SNPs would be required to produce a functional SNP panel. Additional development will be necessary to produce such a panel of highly informative SNPs for clinical use. A real-time PCR SNP assay may ultimately provide more accurate quantification and shortened turnaround time compared to current post-engraftment assays. (*J Mol Diag* 2000, 2:202–208)

Monitoring bone marrow transplant (BMT) patients requires analysis of posttransplant samples to quantify the percentage of engraftment, evaluate for minimal residual disease, and detect early recurrence. Current assays determining percentage of engraftment take advantage of the genetic polymorphisms that exist between individuals. The earliest of these assays, restriction fragment length polymorphism analysis (RFLP)-Southern blot,

used radioactive probes to anneal to large genomic DNA fragments of up to 25 kb.^{1–4} Subsequent assays used the variability of short, repetitive, non-coding sequences in human DNA to differentiate donor from recipient in engraftment specimens. Polymerase chain reaction (PCR) amplification of the DNA encompassing minisatellites, or variable number of tandem repeats (VNTR)^{5–7} and microsatellites, or short tandem repeats (STR)^{5,8–10} results in amplicons of different lengths, depending on the number of repeats within the sequence. However, drawbacks to these techniques exist, including variable test sensitivity due to somewhat subjective comparison of autoradiographic band intensities (in RFLP or VNTR analysis by Southern hybridization) or ethidium-stained band intensities (in PCR-based assays using VNTRs and STRs). The limit of detection of these techniques is variable, ranging from approximately 5 to 10% for Southern-RFLP to 1 to 2% for minisatellite and microsatellite analysis.^{1,5} Finally, many assays require substantial post-PCR analysis such as restriction enzyme digestion, agarose/acrylamide gel electrophoresis, or automated sequence analysis, all of which increase turnaround time.

In this paper, we present an alternate method of engraftment analysis using single nucleotide polymorphisms (SNPs). SNPs are pairs of alleles which vary at a single DNA basepair. They constitute 90% of all polymorphisms in the human genome, occurring at an average rate of 1/1000 bp.¹¹ Unlike the repeating sequences of VNTRs and STRs, SNPs are non-repetitive sequences, and can be found in both coding and non-coding regions of the genome. Interest in SNPs has surged as a result of applications in the mapping of cancer and other disease genes, population genetics, and pharmacogenomics.^{11–14} Databases, both public (National Center for Biotechnology Information) and private (pharmaceutical), have been established to catalog the growing number of identified SNPs.¹¹

The purpose of our experiments was to develop a rapid, highly sensitive method of quantifying bone marrow engraftment in patients without the need for subjec-

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tive interpretation of assay results or post-PCR analysis. We focused on SNPs because of their prevalence and suitability to quantification by fluorogenic oligonucleotide probes. We describe a novel technique using multiplex real-time PCR amplification of a well-characterized, common, biallelic SNP located within the cytochrome P₄₅₀ CYP2C9 gene.¹⁵ We used an assay containing two oligonucleotide reporter probes (allele 1-specific and allele 2-specific) and a detection system that is linear over at least five orders of magnitude.¹⁶

Materials and Methods

Patient Samples, DNA Isolation, and Chimeric Mixes

Archival DNA previously harvested from the peripheral blood (18 cases) or bone marrow (2 cases) of 20 clinically healthy patients was used for this study. DNA isolation was done by NP40 detergent (Amersham, Piscataway, NJ) lysis of cells, sodium dodecyl sulfate/proteinase K digestion, 5 mol/L NaCl extraction, and ethanol wash.¹⁷ DNA used in real-time PCR reactions was further purified by double phenol-chloroform-isoamyl alcohol extraction, ammonium acetate precipitation, and ethanol wash.¹⁸ DNA concentrations were determined spectrophotometrically using the average of A₂₆₀ measurements from two dilutions. Using these concentrations, DNA from two different samples were mixed in various ratios in preparation for Southern and real-time PCR analysis.

CYP2C9 Genotype Assessment by PCR-AvalI-Polyacrylamide Gel Electrophoresis

To identify samples of known genotype, we performed CYP2C9 genotyping on 50 ng of patient DNA using previously reported PCR primers and conditions,¹⁹ in a final reaction volume of 50 μ L. Twenty-five microliters of this product were digested with 12 units of AvalI (New England BioLabs, Beverly, MA) for 8 hours at 37°C, followed by electrophoresis on an 8% acrylamide gel. The primers identified by Stubbs et al,¹⁹ which we have also used, generate a PCR product that does not contain a second, nonpolymorphic AvalI restriction site. The diagnosis of an allele 2 homozygote would, therefore, be susceptible to a false positive in the event of restriction enzyme failure or inhibition. Accordingly, if one were to employ the RFLP assay for clinical use, the primers would have to be redesigned to incorporate such an internal control cut site. To confirm the genotype determined by RFLP analysis, undigested amplicons were also sequenced using a Big Dye sequencing kit (PE Biosystems, Foster City, CA) and products analyzed on a PE Biosystems ABI 310 Gene Sequencer.

Real-Time PCR SNP Analysis at the CYP2C9 Locus

SNP analysis was also performed using a real-time PCR CYP2C9 allelic discrimination TaqMan assay (PE Biosystems), with minor modifications. The assay includes proprietary nonlabeled forward and reverse primers along with two proprietary fluorescent TaqMan oligonucleotide probes (allele 1-specific probe labeled with VIC fluorophore, allele 2-specific probe labeled with FAM (6-carboxy-fluorescein fluorophore)). The VIC and FAM reporter dyes are covalently attached to the 5' terminal base of the two probes and the nonfluorescent quencher dye is attached near the 3' ends. The probes are capable of differentially binding to the amplicons generated during PCR and selectively reporting their respective alleles.²⁰ All PCR reactions were run in triplicate, and contained 100 ng of patient DNA, 12.5 μ L of TaqMan Universal PCR Master Mix, and 2.5 μ L of Allelic Discrimination Mix. Appropriate negative controls were also run. Real-time PCR was performed on an ABI Prism 7700 Sequence Detection System (SDS, PE Biosystems) using the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, and then 40 cycles of amplification (92°C denaturation for 15 seconds, 62°C annealing/extension for 60 seconds). The annealing temperature was empirically determined (data not shown) to promote high binding specificity of the probes without loss of assay sensitivity. For each cycle, the SDS software determines the ΔR_n , which is the normalized (ie, compared to a passive reference fluorophore) fluorescent signal from the VIC- or FAM-labeled probe. For our analysis we used the ΔR_n value after the final cycle, because it proved more reliable than the cycle at which the threshold was crossed (C_T value, data not shown). The $\Delta R_{nFAM}/\Delta R_{nVIC}$ fluorophore signal ratio (hereafter abbreviated FAM/VIC) was calculated for each individual real-time PCR reaction, and means with standard deviations were determined for the triplicate runs.

RFLP-Southern Analysis

Three micrograms of DNA were digested with *HinfI* (New England BioLabs), run on an agarose gel, transferred to a Zeta-Probe nylon membrane (Biorad, Hercules, CA) and hybridized with a ³²P-labeled D1S7 (MS-1) minisatellite probe²¹ using standard techniques.²² The percentage of each patient's DNA was determined using a Molecular Dynamics (Sunnyvale, CA) 445SI PhosphorImager.

Statistical Calculation of Loci Informativity

Two methods for calculating informativity are described (Table 1). In method A, only homozygotes are considered informative, whereas with method B, both homozygotes and heterozygotes are considered informative. For initial calculations, we assumed a theoretical optimal SNP allele frequency of 50% in a population ($p = q = 0.5$). For method A, the probability that a given donor is homozygous for the p allele is 0.25 ($p^2 = 0.50^2$) from the Hardy-

Table 1. Informativity Calculations According to Method

Method used	Informative combinations		Non-informative combinations		Percentage of donor-recipient pairs informative per optimal SNP	Number of loci needed (% cases informative)
	Donor	Recipient	Donor	Recipient		
A (homozygotes informative only)	pp	qq	pp	pq	12.5	25 (96.5%)
	qq	pp	pq	pp		
			qq	pq		
			pq	qq		
			pp	pp		
			qq	qq		
			pq	pq		
B (homozygotes and heterozygotes informative)	pp	qq	pp	pp	62.5	4 (98.0%)
	qq	pp	qq	qq		
	pp	pq	pq	pq		
	pq	pp				
	qq	pq				
	pq	qq				

Weinberg relationship ($p^2 + 2pq + q^2 = 1$). The probability that the recipient is homozygous for the q allele is 0.25, yielding a combined probability of 0.0625. Informativity is also obtained if the donor is qq and the recipient is pp (probability also equal to 0.0625). Therefore when only homozygosity is considered informative, the total informativity of this SNP is twice that value, 0.125 or 12.5%.

In method B, both homozygotes and heterozygotes are considered informative (Table 1). The relative frequencies of each informative genotype are calculated with the same optimal SNP. Together they provide an overall informativity of 62.5%, when the allele frequency of p is 0.5. Similar calculations using methods A and B were repeated for lower p allele (and correspondingly higher q allele) frequencies.

Assuming an informativity of i for each SNP and independent assortment of SNPs, the binomial theorem indicates the probability that a given donor-recipient pair will yield at least one informative locus when n loci are evaluated is:

$$p_n = 1 - (1 - i)^n$$

Assuming an i informativity and assembly of a panel of n SNPs, the probability of having y number of loci be informative is given by the general formula:

$$\text{Pr}(Y = y) = \frac{n!}{y!(n - y)!} (i)^y (1 - i)^{n-y}$$

Results

CYP2C9 SNP Genotyping of Samples

To assess the utility of using SNPs and real-time PCR for engraftment analysis, we first determined the genotype of the SNP at the 440 bp position of the P_{450} CYP2C9 gene in patient samples using the RFLP method described by Stubbins et al.¹⁹ Allele 1 (CYP2C9*1) contains a C at the 440 bp position, whereas allele 2 (CYP2C9*2) contains a

T (Figure 1A). This method entailed PCR amplification of DNA, *Ava*II digestion, 8% polyacrylamide gel electrophoresis, and identification of characteristic band patterns. In our study population of 20 patients we found 4 (20%) heterozygotes, consistent with the 19% heterozygous frequency of this SNP reported in general population studies.^{19,23} All other patients in our study were allele 1 homozygotes; no allele 2 homozygotes were found in our limited population. Results of three representative samples are shown in Figure 1B. Because no internal *Ava*II restriction site is present within the amplified product, the undigested PCR products shown in Figure

Figure 1. CYP2C9 SNP structure and sample characterization/genotyping. **A:** A single nucleotide polymorphism (SNP) in the CYP2C9 gene contains a C (allele 1) or T (allele 2) at the locus. The C→T alteration destroys an *Ava*II (GGACC) site. **B:** A 190-bp fragment containing the SNP was PCR-amplified, digested by *Ava*II, and run on a polyacrylamide electrophoresis gel. The allele 1 sequence contains an *Ava*II site and is digested into 75-bp and 115-bp fragments; the site is lost in allele 2, so this amplicon remains 190-bp after digestion. Heterozygous DNA produces 190-, 115-, and 75-bp fragments. Examples from three different individuals are shown before and after cutting with *Ava*II.

Figure 2. Real-time PCR allelic discrimination of the CYP2C9 SNP. SNPs affect fluorescent probe binding to complementary DNA, allowing discrimination of two alleles. Probes that are a perfect complement to the target DNA hybridize and are digested by the 5'→3' exonuclease activity of *Taq* polymerase, separating VIC or FAM reporter from the quencher. Single base mismatched probes do not bind at this relatively high annealing temperature (non-hybridize), remain intact, and do not report, due to quenching of FAM or VIC fluorescence.

1B were sequenced to confirm their identity (data not shown). These patient samples with defined genotypes were then used for subsequent real-time PCR analysis.

Real-Time PCR Analysis

To demonstrate whether SNPs could be used to accurately report donor/recipient ratios in the post-BMT setting, we created mock mixed chimeras using samples of established genotype. Such DNA mixes are commonly used as accurate representations of cell chimeras in a variety of engraftment analyses.^{1,2,5,8} The TaqMan assay contained two probes that could discriminate alleles differing at only a single base (Figure 2). DNA mixes of two allele 2/allele 1 heterozygous samples were diluted into an allele1 homozygous sample at proportions of 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 3, 1, and 0% heterozygote (equivalent to 50, 45, 40, 35, 30, 25, 20, 15, 10, 5, 2.5, 1.5, 0.5, and 0% allele 2). Real-time PCR was then performed using the conditions detailed above, and two standard curves of % allele 2/% allele 1 versus FAM/VIC ratio were generated (Figure 3). These two parameters showed a linear relationship in both standard curves ($r^2 = 0.987$ and $r^2 = 0.977$), except in the mixes with the lowest percentage of allele 2 (<5%). Thus, changes in the ratio of allele 2 (FAM fluorophore) to allele 1 (VIC fluorophore) in a DNA mix results in a predictable change in the ratio of intensities emitted by their corresponding fluorescent probes after PCR amplification. The plots from both mock chimeras were almost superimposable (within the limits of pipetting and spectrophotometry error), indicating that the standard curve is reproducible and perhaps applicable to other patient mixes as well. If such is the case, generating standard curves for each individual patient may not be necessary.

Assay Validation Compared to Southern Blotting

We validated our assay against a classic BMT engraftment assay, RFLP-Southern blot using a D1S7 minisatellite probe. In this comparison, the same homozygous and heterozygous patient DNA used above were mixed in new proportions and run as blinded unknowns in both the TaqMan SNP and RFLP-Southern blot assays. The FAM/VIC ratio calculated from each mix in the SNP assay was translated to % allele 2/% allele 1 by extrapolation from the consensus standard curve of Figure 3. In the RFLP-

Figure 3. Fluorophore emission after real-time PCR amplification of DNA mixes. Standard curves were generated by mixing DNA from two allele 2 heterozygotes into an allele 1 homozygote in varying proportions, and then performing real-time PCR. The calculated FAM/VIC ratio was plotted with respect to the known percentage of allele 2/percentage of allele 1 in the corresponding mix. Error bars indicate 1 SD for triplicate runs. The standard curves allow extrapolation of % allele 2/% allele 1 (x-axis) in unknown specimens using the measured FAM/VIC ratio (y-axis).

Figure 4. Validation of the real-time PCR SNP assay with the Southern blot assay. The real-time PCR SNP assay (TaqMan) is compared to Southern blot by running mock chimeric mixes of 100, 74, 62, 43, 28, 9, and 0% heterozygous DNA using both techniques, evaluated in a blinded fashion. The correlation coefficient between the two assays is 0.971, and the slope of the regression line is 1.004.

Southern blot assay, % allele 2/% allele 1 was determined by phosphorimager analysis. Overall, the results of the two assays were highly correlated ($r^2 = 0.971$), as shown in Figure 4.

Number of SNPs Required for a Clinical Panel

We developed a statistical model to determine how many SNPs should be included in a screening panel designed to assay for post-BMT engraftment. In our initial calculations, we assumed an optimal informativity for a given SNP, with an allele frequency of 50% ($p = q = 0.5$). We applied the Hardy-Weinberg relationship ($p^2 + 2pq + q^2 = 1$) to determine the frequency of a given genotype. Calculations were done using two different definitions of informativity.

In method A, only homozygotes are considered informative such that only two of the nine possible allele combinations are informative (Table 1). A given pair of individuals is informative if one person is homozygous for one of the alleles and the other is homozygous for the other allele. Under these conditions, we found the total informativity of an optimal theoretical SNP to be 0.125, or 12.5% (see Materials and Methods). If we assume that multiple SNPs would be analyzed before transplantation, how many would be required to find an informative locus in the majority of donor-recipient pairs? Assuming independent inheritance (no linkage disequilibrium) and applying the binomial theorem, we calculated that if only five optimal SNPs are analyzed, only 49% of random donor-recipient pairs will be informative for one of them. For 10 SNPs, 74% will have at least one informative locus, and for a panel of 25 SNPs, 96.5% of pairs will have at least one informative SNP. With a panel of 25 SNPs, the probability of finding no informative alleles is only 3.5%, and

Figure 5. Variation in allele frequency alters the number of SNPs required in a panel. The allele frequency of p was varied from 50% to 2% with a corresponding increase in q . The number of SNP loci that must be tested in each donor and recipient sample in order to be more than 95% certain of finding at least one informative SNP is indicated for various frequencies of the p allele in a population. Data are calculated and graphed using both method A and method B (see Materials and Methods).

the average number of informative SNPs is expected to be about 3 (25×0.125).

In method B, both homozygotes and heterozygotes are considered informative. In this setting, six of the nine possible combinations are informative (Table 1), resulting in an overall informativity of the single optimal SNP of 62.5%. Accordingly, far fewer SNPs would be required to produce a functional panel for monitoring BMT engraftment. Only three SNPs would provide an overall informativity of almost 95%; with four SNPs, 98% of donor-recipient pairs would be informative for at least one SNP. Transplantation between family members would require a larger number of SNPs to be screened to achieve these same levels of informativity. Thus, including informative heterozygotes in these analyses has a profound effect on the number of loci required to achieve a reasonable overall informativity.

Effect of Allele Frequency on SNP Panel Size

The number of SNPs required in a panel is determined in part by the allele frequency of p and q in the population. Using various allele frequencies of 2 to 50%, we have calculated the number of SNPs needed to compose a panel so that at least one of the SNPs would be informative between the donor and recipient 95% of the time (Figure 5). Using method B to define informativity, altering the p allele frequency from 0.20 to 0.50 has very little effect on the number of SNPs required. Method A is more dependent on variations in allele frequency. Allele frequencies below 30% using method A, or below 2% using method B, would require analysis of too many SNPs to be practical.

Discussion

Several assays have been developed previously to follow the transplantation of donor bone marrow into a recipient

to document engraftment and monitor for disease relapse. There are an extensive number of probes available for RFLP-Southern analysis, such that an informative locus can nearly always be found, even in cases involving discrimination of siblings.¹ The typical assays, however, are costly and time-consuming. Techniques exploiting amplification of polymorphisms containing small repeats (minisatellites and microsatellites) are confounded by polymerase slippage and require post-PCR analysis. Unfortunately, microsatellite and minisatellite analyses are not readily analyzable by real-time PCR using fluorescent probes.

In this report we provide evidence that SNPs can be used to monitor BMT engraftment. Nearly any SNP can be used in this assay; our choice of the CYP2C9 SNP was based solely on the frequency of the polymorphism and the availability of the primers and probes in a commercially available system. For clinical use, multiple SNPs would have to be evaluated as a panel in the donor and recipient by the real-time PCR assay, before transplantation, to identify an informative locus. How one defines informativity (homozygotes only *versus* both homozygotes and heterozygotes) has a profound effect on the number of SNP loci required for analysis. In method A, a panel of 23 highly informative SNPs would need to be run to be >95% certain that at least one SNP was informative, whereas in method B, only about 4 such loci are required to achieve a similar level of confidence. Method B is also much less sensitive to allele frequencies lower than the 50% theoretical optimum. Once an adequate SNP locus is identified which distinguishes the donor from the recipient, all posttransplant assays would then be run using only one of the informative SNPs identified.

The linear relationship between the percentage of allele 2 present in a chimeric mix and the FAM/VIC emission ratio becomes curvilinear at the lowest levels of percentage of allele 2 in Figure 3. This may represent nonspecific binding of some FAM probe to allele 1 DNA due to a relative excess of allele 1 DNA. Additional experiments with mixes of known allele 2 homozygotes will need to be performed with optimal probes to further define the actual limit of detection of the SNP real-time PCR approach. With further optimization, the SNP based real-time PCR assay may attain the limit of detection of microsatellite and minisatellite based assays currently in use.

BMT engraftment assessment using the real-time PCR SNP assay described herein may prove to be an improvement over current engraftment assays. Because the ABI 7700 can discriminate probe signals and measure them over a range of several orders of magnitude,¹⁶ relatively small amounts of recipient DNA should be detectable in a posttransplant marrow specimen. Fortunately, our data suggest that standard curves may extrapolate to other patients (ie, will not be patient-specific), though this will need to be confirmed with additional patient samples. Furthermore, the assay requires only minute amounts of DNA (100 ng, as typically obtained in cytopenic bone marrow specimens), does not require donor-recipient sex mismatch or radioactivity, and circumvents differential amplification of unequal length alleles. Finally, a real-time PCR-based assay has the po-

tential to reduce technologist time, improve turnaround time, and consequently reduce medical cost.

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