Clinica Chimica Acta 445 (2015) 101–106

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Rapid single nucleotide polymorphism based method for hematopoietic chimerism analysis and monitoring using high-speed droplet allele-specific PCR and allele-specific quantitative PCR

Chiaki Taira [a], Kazuyuki Matsuda [a,*], Akemi Yamaguchi [b], Masayuki Uehara [b], Mitsutoshi Sugano [a], Nobuo Okumura [c], Takayuki Honda [a]

[a] Department of Laboratory Medicine, Shinshu University Hospital, Matsumoto, Japan
[b] Core Technology Development Center, Seiko Epson Corporation, Fujimi, Japan
[c] Laboratory of Clinical Chemistry and Immunology, Department of Biomedical Laboratory Sciences, School of Health Sciences, Shinshu University, Matsumoto, Japan

ARTICLE INFO

Article history:
Received 8 February 2015
Received in revised form 27 February 2015
Accepted 7 March 2015
Available online 20 March 2015

Keywords:
HSCT
SNP
Droplet-AS-PCR
AS-qPCR

ABSTRACT

Background: Chimerism analysis is important for the evaluation of engraftment and predicting relapse following hematopoietic stem cell transplantation (HSCT). We developed a chimerism analysis for single nucleotide polymorphisms (SNPs), including rapid screening of the discriminable donor/recipient alleles using droplet allele-specific PCR (droplet-AS-PCR) pre-HSCT and quantitation of recipient DNA using AS-quantitative PCR (AS-qPCR) following HSCT.
Methods: SNP genotyping of 20 donor/recipient pairs via droplet-AS-PCR and the evaluation of the informativity of 5 SNP markers for chimerism analysis were performed. Samples from six follow-up patients were analyzed to assess the chimerism via AS-qPCR. These results were compared with that determined by short tandem repeat PCR (STR-PCR).
Results: Droplet-AS-PCR could determine genotypes within 8 min. The total informativity using all 5 loci was 95% (19/20). AS-qPCR provided the percentage of recipient DNA in all 6 follow-up patients without influence of the stutter peak or the amplification efficacy, which affected the STR-PCR results.
Conclusion: The droplet-AS-PCR had an advantage over STR-PCR in terms of rapidity and simplicity for screening before HSCT. Furthermore, AS-qPCR had better accuracy than STR-PCR for quantification of recipient DNA following HSCT. The present chimerism assay compensates for the disadvantages of STR-PCR and is readily performable in clinical laboratories.

1. Introduction

Hematopoietic stem cell transplantation (HSCT) has become a well-established treatment for patients suffering from malignant disorders and other disorders such as aplastic anemia, congenital immunodeficiency, or inborn errors of metabolism. After transplantation, determination of the extent of chimerism, i.e., the ratio of recipient-origin cells to donor-origin cells, is important for detecting early signs of graft rejection, preventing relapse of the underlying disease, and monitoring of minimal residual disease (MRD) [1,2].

Various polymerase chain reaction (PCR) methods using polymorphic DNA loci, such as variable number tandem repeats and short tandem repeats (STRs), have been developed for assessment of chimerism because PCR amplification is a rapid, convenient, and sensitive technique. In particular, analysis of polymorphic STR loci by PCR (STR-PCR) is widely used for chimerism assessment because of the high informativity of STRs. Multiplex STR-PCR using several STR loci shows a high discrimination rate of >95% between donor and recipients [3–6]. However, chimerism assessment based on STR-PCR analyses requires dilution of the STR-PCR amplicon before fragment analysis using capillary electrophoresis with a DNA sequencer [7] and is affected by the stutter peaks produced during PCR amplification [8,9], which reduces the quantification accuracy of this method.

Recently, analyses based on real-time PCR using single nucleotide polymorphisms (SNPs) have been reported as alternative methods with more accurate quantification [10–13]. For detection of an SNP or a single nucleotide mutation, allele-specific PCR (AS-PCR) methods based on an amplification refractory mutation system or a mismatch amplification mutation assay have been developed [14–16]. AS-quantitative

Abbreviations: HSCT, hematopoietic stem cell transplantation; MRD, minimal residual disease; STR-PCR, short tandem repeat polymerase chain reaction; SNP, single nucleotide polymorphism; AS-PCR, allele-specific polymerase chain reaction; AS-qPCR, allele-specific quantitative polymerase chain reaction; droplet-AS-PCR, droplet allele-specific polymerase chain reaction; MAF, minor allele frequency.

* Corresponding author at: Department of Laboratory Medicine, Shinshu University Hospital, 3-1-1, Asahi, Matsumoto 390-8621, Japan. Tel.: +81 263 37 2802; fax: +81 263 34 5316.
E-mail address: kmatsuda@shinshu-u.ac.jp (K. Matsuda).

http://dx.doi.org/10.1016/j.cca.2015.03.018
0009-8981/

PCR (AS-qPCR) using a fluorescence probe such as TaqMan probe can quantify the target allele, indicating that AS-qPCR for SNPs has potential to be applied to a chimerism assay following HSCT [10,12].

In the present study, we developed a chimerism analysis method based on AS-PCR/AS-qPCR for SNPs. The method involves rapid screening of the discriminable donor/recipient alleles by using high-speed droplet AS-PCR (droplet-AS-PCR) pre-HSCT, and quantitation of recipient DNA by using AS-qPCR following HSCT.

2. Materials and methods

2.1. Patients

A total of 40 peripheral blood (29), cord blood (7), or bone marrow (4) samples from 20 donor/recipient pairs (all pairs are Japanese) that underwent HSCT were used for SNP genotyping by droplet-AS-PCR, and for the evaluation of the informativity of the SNP markers for chimerism analysis. Six patients available for follow-up bone marrow samples post-HSCT (Table 1) were analyzed to assess the chimerism by AS-qPCR, and the results were compared with those obtained by STR-PCR. This study was approved by the Institutional Review Board of Shinshu University.

2.2. Genomic DNA extraction

Extraction of genomic DNA from peripheral blood or bone marrow was performed using a QIAamp DNA blood mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions.

2.3. Selection of SNP loci and design of primers and probes

We selected SNPs loci with high minor allele frequency (MAF), which are located on different regions of chromosomes according to the previous studies [12,17,18] and our experiment. In the preliminary study using unrelated 8 pairs, 5 SNPs provided 100% of probability in identifying informative loci in the all 8 pairs. In this study, we used the 5 SNPs loci (Table 2). For each allele, we designed allele-specific primers that included an SNP-matched nucleotide at the 3′-end, a template-mismatched nucleotide at the −2 position from the 3′-end, a TaqMan probe labeled with fluorescein amidite at the 5′-end nucleotide, and a quencher (Black Hole Quenchers, Sigma-Aldrich, St, Louis, MO, USA) at the 3′-end nucleotide. The sequences of primers and probes used in this study are listed in Table 2.

2.4. Screening of informative SNP loci by droplet-AS-PCR

SNP genotyping was performed using a novel high-speed droplet-PCR machine (Seiko Epson, Nagano, Japan) [18–20]. The droplet-AS-PCR mixture contained genomic DNA (30–50 ng/μL), Platinum Taq DNA polymerase (0.1 U/uL), Life Technologies; Grand Island, NY, USA), primer (800 nmol/L; designed as described above), TaqMan probe (300 nmol/L), and a reaction buffer (10 μL) composed of Tris–HCl, pH 9.0, KCl, and MgCl2. The reaction conditions were as follows: 95 °C for 10 s and 35 cycles at 95 °C for 4 s and 60–66 °C for 8 s. The droplet-AS-PCR assays for the 5 SNP loci were performed in duplicate. We determined an arbitrary standard to be 6.7 for judging positive amplification according to our previous report [18]. In this study, we defined informative SNP loci as those presenting a heterozygous genotype in the recipient and a homozygous genotype in the donor and/or a homozygous genotype in the recipient and an alternate homozygous genotype in the donor.

2.5. STR-PCR

The amplified fragments from each allele at 5 STR loci (FGA, D5S818, SE33, VWF, and D18S51) were analyzed using Gene Mapper analysis software, as described previously [21] (Table 3). The percentage of recipient DNA was calculated using the Recipient–Shared–Donor (RSD) code according to the equation of Lion and Watzinger [22,23]. In the RSD code, DDRR, %recipient = (HR1 + HR2) / (HR1 + HR2 + HD1 + HD2); SDR, %recipient = HR / (HR + HD); and DRR, %recipient = (HR1 + HR2) / (HR1 + HR2 + HD), where H is the peak height of the recipient alleles (HR, HR1, HR2) and the donor alleles (HD, HD1, HD2) and S is the number of shared alleles between the donor and recipient. Alleles potentially affected by stutter peak formation (coded as lowercase letters) are generally disregarded in the formulas for the calculation of chimerism; however, in this study, the percent of recipient DNA in dRdR and RddR was calculated as follows: (HR1 + HR2) / (HR1 + HR2 + Hd1 + Hd2).

2.6. AS-qPCR

The AS-qPCR mixture contained 20–50 ng genomic DNA, 1× TaqMan Universal PCR Master Mix (Applied Biosystems; Foster City, CA, USA), 0.5 μmol/L of each primer, and 0.25 μmol/L of TaqMan probe in a total volume of 50 μL. AS-qPCR was performed using an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). The reaction conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, and 50 cycles of 95 °C for 15 s and 60 °C for 1 min. As an internal control

Table 1
Summary of 6 patients.

Patient no.	R/D	Age (year)/sex	Race	Diagnosis	Karyotype	Conditioning regimen	All-HSCT
1	R	0/F	Japanese	FHL	46,XX [20]	TBI/Flu/Cy	uCBT
	D	N.A./M	Japanese				
2	R	8/M	Japanese	ALL	46,XY [17].ish t(9;22)(q34;q11.2)	TBI/Flu/Cy	uCBT
	D	N.A./M	Japanese				
3	R	22/F	Japanese	MDS	47,XX,+11[2]/46,XX[13]	TBI/AraC/Cy	uCBT
	D	N.A./M	Japanese				
4	R	51/F	Japanese	AML	46,XX [20]	Flu/Bu	uBMT
	D	N.A./M	Japanese				
5	R	54/M	Japanese	CMMoL	46,XY [20]	Flu/AraC	uBMT
	D	N.A./F	Japanese				
6	R	55/M	Japanese	AML	46,XY [20]	Flu/Bu	uBMT
	D	N.A./F	Japanese				

R, recipient; D, donor; FHL, familial hemophagocytic lymphohistiocytosis; ALL, acute lymphoblastic lymphoma; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; CMMoL, chronic myelomonocytic leukemia; AML, acute myelogenous leukemia; TBI, total body irradiation; Flu, fludarabine; Cy, cyclophosphamide; AraC, cytarabine; Bu, busulfan; all-HSCT, allogeneic hematopoietic stem cell transplantation; uCBT, unrelated cord blood transplantation; uBMT, unrelated bone marrow transplantation. All donors are Bank Donor, so age is not available (N.A.). Karyotyping was expressed according to an International System for Human Cytogenetic Nomenclature (ISCN) 2013.

Table 2
Sequences of primers and probes for droplet-AS-PCR and AS-qPCR.

dbSNP ID (MAFs)	Chromosome location	Nucleotide variation	Primers (5′–3′)	Probes (5′–3′)
rs2385512 (0.3241)	1	A G	F: GAATATTATCTGGCCAAGCAGGG**A**A**A** F: GAATATTATCTGGCCAAGCAGGG**G**A**G** Internal-F: CCATGAATATTATCTGGCCAAGCAGG R: GTCCCACAAGCAGGGCTGTC	FAM-TGGGCCCTACACCCTACTCTCCTGTGGG-BHQ
rs3769393 (0.3029)	2	A G	R: CCTCTAGCTCCTTAATGGATAA**A**G**T** R: CCTCTAGCTCCTTAATGGATAA**T**G**C** Internal-R: CATCTTCCTCTAGCTCCTTAATGGATA F: GCCTTGAATACATCTTCATTTTAAATC	FAM-TGCATCTTGAACACTGAAAACCCCATGACT-BHQ
rs748235 (0.2212)	10	A G	F: GCTCATTTCCACCTCACC**T**A**A** F: GCTCATTTCCACCTCACC**T**A**G** Internal-F: CTGGCTCATTTCCACCTCAC R: GTGCGTCTGCTTCCTCAGC	FAM-CCGCATCCTCTTCTTCACCTCCAGCA-BHQ
rs1386718 (0.3219)	11	A G	F: GTGGCAATGGCAGAAGTC**G**A**A** F: GTGGCAATGGCAGAAGTC**G**A**G** R: ACAGTTCTGAGCAAGAACCTTG	FAM-TCCAGGGCTCAACTCAGAGTCCAAGCA-BHQ
rs12438539 (0.4185)	15	G T	F: CTTCCTCCCTGCTCTAGT**A**G**G** F: CTTCCTCCCTGCTCTAGT**T**G**T** Internal-F: GAAGCTTCCTCCCTGCTCTAG R: GGGAGCTGGGAGGGTAGG	FAM-CATAGCTCAGAGCTGGGCTTCCCTCCTCCCACCCT-BHQ

Polymorphic nucleotides are given in bold, and mismatched nucleotides introduced are bold underlined. Internal of primers: as an internal control in relative quantitation by AS-qPCR, we used locus-individualized endogenous control with genomic position overlapping with the sequence of the respective informative SNP according to the report by Gineikiene et al [12]. The locus of rs1386718 was used for only the discrimination of donor/recipient pairs, so internal primer has not shown. MAFs, minor allele frequencies by the dbSNP database [17]. FAM, fluorescein amidite; BHQ, black hole quencher.

in relative quantitation by AS-qPCR, we used locus-individualized endogenous controls with a genomic position overlapping the sequence of the respective informative SNPs, according to Gineikiene et al. [12]. Relative levels of recipient DNA in the 6 patients post-HSCT were calculated based on the $2^{-\Delta\Delta Ct}$ method [13]. The fraction of recipient genotype pre-HSCT was defined as 100%, and the percentage of the fraction for each informative SNP post-HSCT was calculated. The detection limit of our AS-qPCR was determined to be 1.0% using 10-fold serial dilutions of genomic DNA that could distinguish between the recipient and donor.

3. Results

3.1. Screening of informative SNP loci by droplet-AS-PCR

The droplet-AS-PCR could determine the genotype of all the 5 SNP loci within 8 min. The informativity of the 5 SNP loci used in this study was 35% (7/20) for rs2385512, 50% (10/20) for rs3769393, 45% (9/20) for rs748235, 5% (1/20) for rs1386718, and 50% (10/20) for rs12438539. For the 20 donor/recipient pairs studied, the total informativity using the 5 loci was 95% (19/20). Half of these 20 donor–recipient pairs had 2 or more informative SNP loci that could be used to discriminate between the donor and recipient (Fig. 1).

3.2. Comparison of the percentage of recipient DNA detected by AS-qPCR and STR-PCR

AS-qPCR was performed using samples from 6 patients whose chimerism status had been monitored using STR-PCR. The informative SNP and STR loci used in each of the 6 patients are listed in Table 4.

The percentages of recipient DNA detected by AS-qPCR and STR-PCR are shown in Fig. 2. In patients 3 to 6, changes in the percentages of recipient DNA following HSCT were concordant using AS-qPCR and STR-PCR. In patient 1, in 3.5 months following HSCT, the percentages of recipient DNA using AS-qPCR for rs238512 and rs12438539 were 2.91% and 2.04%, respectively, whereas the amount of recipient DNA using STR-PCR was under the detection limit. In patient 2, the percentages of recipient DNA using AS-qPCR were close to the detection limit at all measurement points following HSCT. On the other hand, 1.25–2.16% recipient DNA was detected using STR-PCR at all measurement points (except for at 6 months following HSCT).

4. Discussion

Assessment of chimerism is critical for monitoring and determining the engraftment of donor cells and for predicting the imminent relapse of the original disease in patients following HSCT, especially for those

Table 3
Characteristics of markers analyzed by STR-PCR.

Locus designation	Chromosome location	Common sequence motif	Product size (bp)	Primer sequence, 5′–3′
FGA	4q28	(TCTT)n	196-352	Forward: FAM-CCATAGGTTTTGAACTCACAG Reverse: CTTCTCAGATCCTCTGACAC
D5S818	5q21-q31	(AGAT)n	133-169	Forward: FAM-AGGGTGATTTTCCTCTTTGGT Reverse: TGATTCCAATCATAGCCACA
SE33	6q14	(AAAG)n	197-343	Forward: FAM-AATCTGGGCGACAAGAGTGA Reverse: ACATCTCCCCTACCGCTATA
VWF	12p12-pter	(ATCT)n	102-132	Forward: FAM-AGCTATATATCTATTTATCAT Reverse: AGATACATACATAGATATAGG
D18S51	18q21	(AGAA)n	286-373	Forward: HEX-TTCTTGAGCCCAGAAGGTTA Reverse: ATTCTACCAGCAACAACACAAATAAAC

FAM, fluorescein amidite; HEX, hexachloro fluorescein.

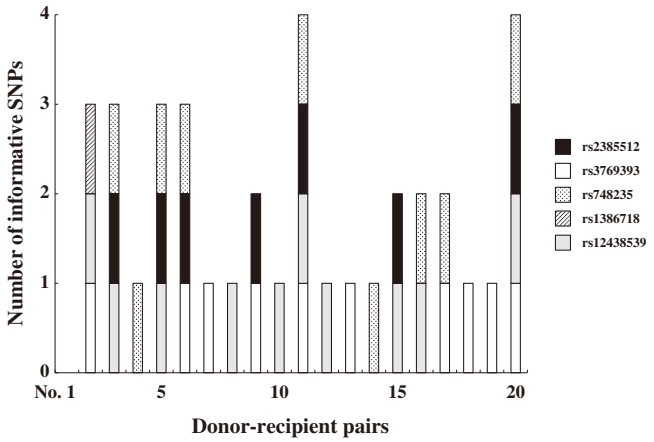

Fig. 1. The number of informative SNP loci identified in donors and recipients of 20 donor/recipient pairs. The histogram shows the number of informative SNP loci identified in donors and recipients. In this study, we defined informative SNP loci as those presenting a heterozygous genotype in the recipient and a homozygous genotype in the donor and/or a homozygous genotype in the recipient and an alternate homozygous genotype in the donor.

receiving a nonmyeloablative conditioning regimen, donor lymphocyte infusions, or second transplantation [24,25]. In the present study, we developed a rapid SNP-based chimerism analysis method involving SNP genotyping by droplet-AS-PCR pre-HSCT samples and quantitation of recipient DNA using AS-qPCR for SNP genotype following HSCT.

SNPs, as well as STRs, are genetic polymorphic markers that are useful for discriminating between donor and recipient alleles. The region of genetic markers able to discriminate between donor and recipient alleles must be selected (i.e., genotype screening) prior to HSCT when using in either the STR-PCR or AS-PCR/AS-qPCR methods. The present droplet-AS-PCR method could determine these genotypes within 8 min. Owing to favorable features such as the one-step real-time PCR protocol and the droplet-PCR machine, as well as the absence of need for post-amplification manipulation and the remarkable reduction in the time required to change the temperature of the heat block [18–20], the present droplet-AS-PCR method could achieve rapid SNP genotyping that is applicable for screening discriminable donor/recipient alleles prior to HSCT.

In general, the discrimination power of SNP loci is lower than that of STR loci. Therefore, although a chimerism assay using numerous SNPs loci is possible, it is not a practical solution for use in most clinical laboratories. The probability with SNP-PCR in identifying informative locus is varied; 92.6% using 10 SNPs [11], 99.6% using 6 SNPs [12], and 95% using 5 SNPs in this study. We used the combination of the 5 SNPs capable to achieve 100% of probability in discrimination of donor and recipient in the limited number of cases according to our preliminary study. However, in this study, one pair among the 20 cases could not be discriminated. MAFs of SNPs are also varied among race [17,26]. It may be difficult to achieve panels of SNPs with constant 100% of probability among various races and many cases. For clinical use of SNP-PCR, the selection and/or combination of SNPs are important to increase the probability. To apply chimerism assessment to all patients following HSCT as possible, setting of several methods including quantifiable SNP-PCR, STR-PCR, and fluorescent *in situ* hybridization of sex chromosomes (XY-FISH) is requisite.

We previously reported the use of AS-qPCR to quantify single nucleotide mutations in hematological malignancies [27–29] and demonstrated that the method was able to detect mutant DNAs when the recipient DNA was less than 5% or negative [29]. The present AS-qPCR method for SNP detection showed 1.0% sensitivity and provided a chimerism assay with high specificity and sensitivity that was better or equal to that of STR-PCR [21]. The AS-qPCR method for mutation detection is useful to evaluate MRD for patients with a specific mutation in a disease-relevant gene; however, because the locus and types of mutations are variable among diseases or patients, the screening process for the mutation is a time-consuming task and the design of the primers and probes for the mutation needs to be performed individually for each disease or each patient. In contrast to mutations, the loci and types of SNPs are already known. Therefore, once the primers and probes for SNPs are designed, droplet-AS-PCR or AS-qPCR for the SNPs is subsequently applicable to all patients for screening the discriminable donor/recipient alleles and monitoring of chimerism.

In addition to the broader utility of the AS-qPCR method for SNPs than that for mutations, AS-qPCR for SNPs can more accurately quantify the chimerism than STR-PCR, in which the difference in amplification efficiency or the overlay of stutter peaks for donor/recipient STR alleles hamper the accurate measurement of chimerism, as seen in patients 1, 2, and 4 in the present study (Fig. 2).

If the disease-specific markers used for MRD assessment disappear during follow-up, accurate MRD evaluation cannot be performed. Therefore, chimerism analyses can be used to detect autologous DNAs as a complement to MRD assessment. Additional chromosomal evolution during disease progression sometimes occurs, resulting in dynamic chromosomal loss, including the regions harboring the individual discriminable SNPs or STR loci. Therefore, the simultaneous evaluation of several SNPs and STR loci should be performed to avoid obtaining false negative results.

In conclusion, the droplet-AS-PCR method showed an advantage over STR-PCR in terms of rapidity and simplicity of screening the discriminable donor/recipient alleles before HSCT. Furthermore, AS-qPCR for SNPs to quantify the recipient DNA following HSCT had better accuracy than STR-PCR. The present chimerism assay including rapid genotyping by droplet-AS-PCR and accurate quantitation of recipient

Table 4
The informative SNP and STR loci used in the 6 patients.

Patient no.		SNP genotypes					STR loci			
	R/D	rs2385512	rs3769393	rs748235	rs1386718	rs12438539		peak size (bp)		RSD code
1	R	AG	AG	AG	–	TT	SE33	258	282	S8D16R
	D	GG	GG	AA	–	GG		258	266	
2	R	AA	AG	AG	–	–	FGA	196	208	d4R8d4R
	D	GG	GG	AA	–	–		192	204	
3	R	–	–	AG	–	–	FGA	180	204	R16d4d4R
	D	–	–	GG	–	–		196	200	
4	R	–	AG	GG	–	–	vWF	120	132	D4D20R12R
	D	–	AA	AA	–	–		96	100	
5	R	GG	–	AG	–	TT	D5S818	144	156	S4D8R
	D	AA	–	GG	–	GG		144	148	
6	R	–	–	–	–	GT	D18S51	320	336	D8R16R
	D	–	–	–	–	GG		312		

R, recipient; D, donor; RSD code, recipient–shared–donor code. [22,23].

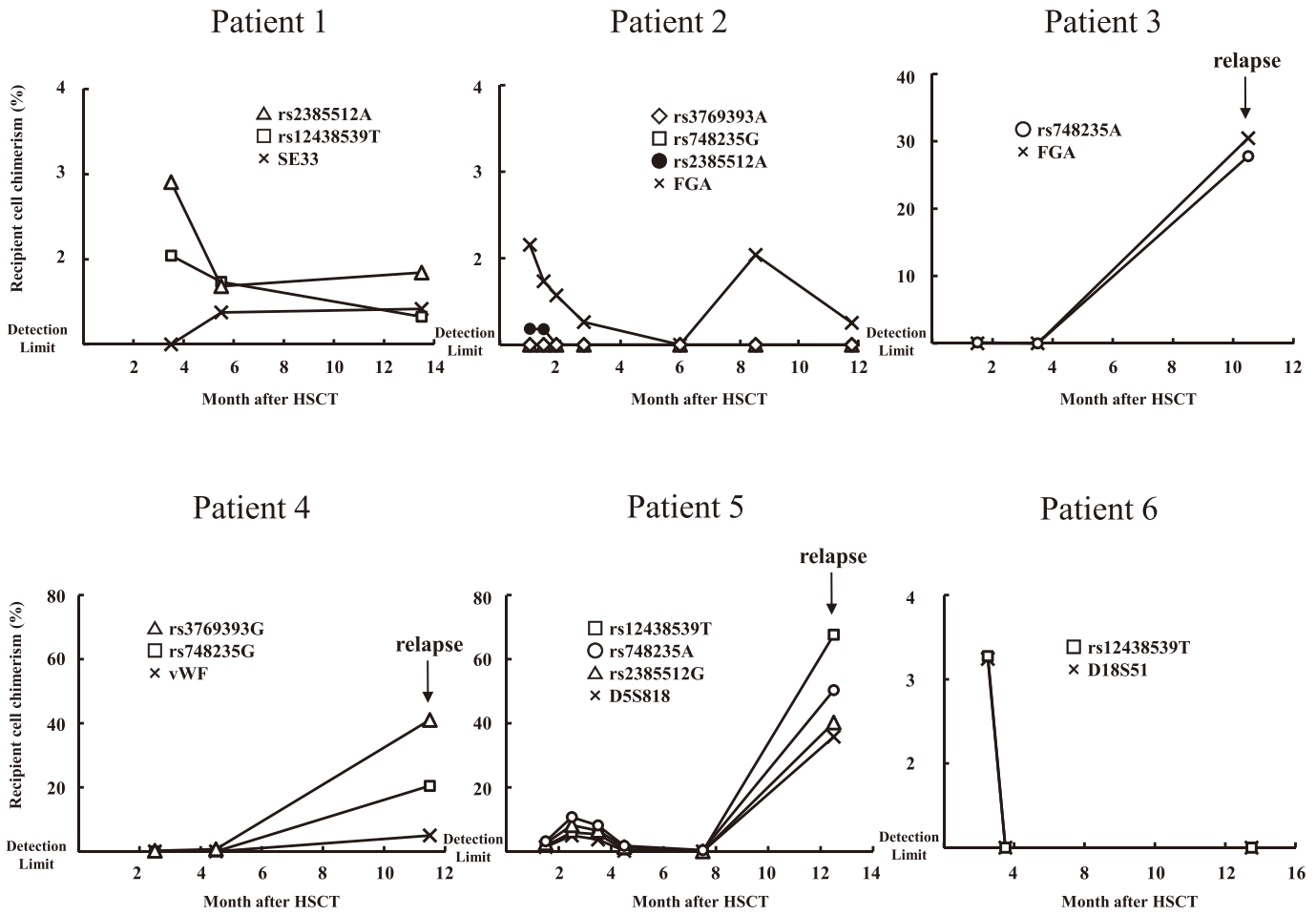

Fig. 2. Comparison of chimerism detected by AS-qPCR with that detected by STR-PCR following HSCT. The percentage of recipient DNA detected by AS-qPCR was compared with that detected by STR-PCR. The quantity of the target SNP alleles using genomic DNA was normalized to the level of a locus-individualized endogenous control with a genomic position overlapping the sequence of the respective informative SNP [12]. The percentage of recipient DNA detected by STR-PCR was calculated based on the respective donor and recipient peak heights according to the equation of Lion and Watzinger [22,23].

DNA by AS-qPCR compensates for disadvantages in STR-PCR and is readily performable in clinical laboratories.

References

[1] Liesveld JL, Rothberg PG. Mixed chimerism in SCT: conflict or peaceful coexistence? Bone Marrow Transplant 2008;42:297–310.
[2] Andreani M, Testi M, Lucarelli G. Mixed chimerism in haemoglobinopathies: from risk of graft rejection to immune tolerance. Tissue Antigens 2014;83:137–46.
[3] Odriozola A, Riancho JA, Mijares V, Nuñez-Cespedes J, Zarrabeitia MT. Chimerism detection by short tandem repeat analysis when donor and recipient genotypes are not known. Clin Chim Acta 2012;413:548–51.
[4] Thiede C, Florek M, Bornhäuser M, Ritter M, Mohr B, Brendel C, et al. Rapid quantification of mixed chimerism using multiplex amplification of short tandem repeat markers and fluorescence detection. Bone Marrow Transplant 1999;23:1055–60.
[5] Nollet F, Billiet J, Selleslag D, Criel A. Standardisation of multiplex fluorescent short tandem repeat analysis for chimerism testing. Bone Marrow Transplant 2001;28:511–8.
[6] Buño I, Nava P, Simón A, González-Rivera M, Jiménez JL, Balsalobre P, et al. A comparison of fluorescent in situ hybridization and multiplex short tandem repeat polymerase chain reaction for quantifying chimerism after stem cell transplantation. Haematologica 2005;90:1373–9.
[7] Kreyenberg H, Hölle W, Möhrle S, Niethammer D, Bader P. Quantitative analysis of chimerism after allogeneic stem cell transplantation by PCR amplification of microsatellite markers and capillary electrophoresis with fluorescence detection: the Tuebingen experience. Leukemia 2003;17:237–40.
[8] Walsh PS, Fildes NJ, Reynolds R. Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA. Nucleic Acids Res 1996;24:2807–12.
[9] Chen DP, Tseng CP, Tsai SH, Wu TL, Chang PY, Sun CF. Systematic analysis of stutters to enhance the accuracy of chimerism testing. Ann Clin Lab Sci 2008;38:264–72.
[10] Maas F, Schaap N, Kolen S, Zoetbrood A, Buño I, Dolstra H, et al. Quantification of donor and recipient hemopoietic cells by real-time PCR of single nucleotide polymorphisms. Leukemia 2003;17:621–9.
[11] Koldehoff M, Steckel NK, Hlinka M, Beelen DW, Elmaagacli AH. Quantitative analysis of chimerism after allogeneic stem cell transplantation by real-time polymerase chain reaction with single nucleotide polymorphisms, standard tandem repeats, and Y-chromosome-specific sequences. Am J Hematol 2006;81:735–46.
[12] Gineikiene E, Stoskus M, Griskevicius L. Single nucleotide polymorphism-based system improves the applicability of quantitative PCR for chimerism monitoring. J Mol Diagn 2009;11:66–74.
[13] Chen DP, Tseng CP, Wang WT, Wang MC, Tsai SH, Sun CF. Real-time biallelic polymorphism-polymerase chain reaction for chimerism monitoring of hematopoietic stem cell transplantation relapsed patients. Clin Chim Acta 2011;412:625–30.
[14] Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, et al. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res 1989;17:2503–16.
[15] Cha RS1, Zarbl H, Keohavong P, Thilly WG. Mismatch amplification mutation assay (MAMA): application to the c-H-ras gene. PCR Methods Appl 1992;2:14–20.
[16] Glaab WE, Skopek TR. A novel assay for allelic discrimination that combines the fluorogenic 5′ nuclease polymerase chain reaction (TaqMan) and mismatch amplification mutation assay. Mutat Res 1999;430:1–12.
[17] The dbSNP database. http://www.ncbi.nlm.nih.gov/SNP/.
[18] Taira C, Matsuda K, Yamaguchi A, Sueki A, Koeda H, Takagi F, et al. Novel high-speed droplet-allele specific-polymerase chain reaction: application in the rapid genotyping of single nucleotide polymorphisms. Clin Chim Acta 2013;424:39–46.
[19] Sueki A, Matsuda K, Taira C, Yamaguchi A, Koeda H, Takagi F, et al. Rapid detection of PML-RARA fusion gene by novel high-speed droplet-reverse transcriptase-polymerase chain reaction: possibility for molecular diagnosis without lagging behind the morphological analyses. Clin Chim Acta 2013;415:276–8.
[20] Matsuda K, Yamaguchi A, Taira C, Sueki A, Koeda H, Takagi F, et al. A novel high-speed droplet-polymerase chain reaction can detect human influenza virus in less than 30 min. Clin Chim Acta 2012;413:1742–5.

[21] Matsuda K, Yamauchi K, Tozuka M, Suzuki T, Sugano M, Hidaka E, et al. Monitoring of hematopoietic chimerism by short tandem repeats, and the effect of CD selection on its sensitivity. Clin Chem 2004;50:2411–4.
[22] Watzinger F, Lion T, Steward C, Eurochimerism consortium. The RSD code: proposal for a nomenclature of allelic configurations in STR-PCR-based chimerism testing after allogeneic stem cell transplantation. Leukemia 2006;20:1448–52.
[23] Lion T, Watzinger F, Preuner S, Kreyenberg H, Tilanus M, de Weger R, et al. The EuroChimerism concept for a standardized approach to chimerism analysis after allogeneic stem cell transplantation. Leukemia 2012;26:1821–8.
[24] Mickelson DM, Sproat L, Dean R, Sobecks R, Rybicki L, Kalaycio M, et al. Comparison of donor chimerism following myeloablative and nonmyeloablative allogeneic hematopoietic SCT. Bone Marrow Transplant 2011;46:84–9.
[25] Chang YJ, Huang XJ. Donor lymphocyte infusions for relapse after allogeneic transplantation: when, if and for whom? Blood Rev 2013;27:55–62.
[26] The JSNP DATABASE. http://snp.ims.u-tokyo.ac.jp/.
[27] Matsuda K, Sakashita K, Taira C, Tanaka-Yanagisawa M, Yanagisawa R, Shiohara M, et al. Quantitative assessment of PTPN11 or RAS mutations at the neonatal period and during the clinical course in patients with juvenile myelomonocytic leukaemia. Br J Haematol 2010;148:593–9.
[28] Taira C, Matsuda K, Kamijyo Y, Sakashita K, Ishida F, Kumagai T, et al. Quantitative monitoring of single nucleotide mutations by allele-specific quantitative PCR can be used for the assessment of minimal residual disease in patients with hematological malignancies throughout their clinical course. Clin Chim Acta 2011;412:53–8.
[29] Taira C, Matsuda K, Saito S, Sakashita K, Sugano M, Okumura N, et al. Application of allele-specific quantitative PCR using genomic DNA to monitor minimal residual disease based on mutant gene levels following allogeneic hematopoietic stem cell transplantation in patients with hematological malignancies: comparison of mutant levels with autologous DNA percentage by short tandem repeat-PCR. Clin Chim Acta 2012;413:516–9.