Leukemia (2012) 1 – 8

www.nature.com/leu

ORIGINAL ARTICLE

The EuroChimerism concept for a standardized approach to chimerism analysis after allogeneic stem cell transplantation

T Lion[1], F Watzinger[1], S Preuner[1], H Kreyenberg[2], M Tilanus[3], R de Weger[4], J van Loon[4], L de Vries[4], H Cavé[5], C Acquaviva[5], M Lawler[6], M Crampe[6], A Serra[7], B Saglio[7], F Colnaghi[8], A Biondi[8], JJM van Dongen[9], M van der Burg[9], M Gonzalez[10], M Alcoceba[10], G Barbany[11], M Hermanson[11], E Roosnek[12], C Steward[13], J Harvey[14], F Frommlet[15] and P Bader[2] on behalf of the EuroChimerism Consortium (EU-Project number: QLG1-CT-2002-01485)

Hematopoietic stem cell transplantation is becoming an increasingly important approach to treatment of different malignant and non-malignant disorders. There is thus growing demand for diagnostic assays permitting the surveillance of donor/recipient chimerism posttransplant. Current techniques are heterogeneous, rendering uniform evaluation and comparison of diagnostic results between centers difficult. Leading laboratories from 10 European countries have therefore performed a collaborative study supported by a European grant, the EuroChimerism Concerted Action, with the aim to develop a standardized diagnostic methodology for the detection and monitoring of chimerism in patients undergoing allogeneic stem cell transplantation. Following extensive analysis of a large set of microsatellite/short tandem repeat (STR) loci, the EuroChimerism (EUC) panel comprising 13 STR markers was established with the aim to optimally meet the specific requirements of quantitative chimerism analysis. Based on highly stringent selection criteria, the EUC panel provides multiple informative markers in any transplant setting. The standardized STR-PCR tests permit detection of donor- or recipient-derived cells at a sensitivity ranging between 0.8 and 1.6%. Moreover, the EUC assay facilitates accurate and reproducible quantification of donor and recipient hematopoietic cells. Wide use of the European-harmonized protocol for chimerism analysis presented will provide a basis for optimal diagnostic support and timely treatment decisions.

Leukemia advance online publication, 3 April 2012; doi:10.1038/leu.2012.66

Keywords: microsatellites; short tandem repeats; alleles; PCR

INTRODUCTION

Quantitative monitoring of recipient- and donor-derived cells by molecular methods has become an indispensable diagnostic tool in the surveillance of patients undergoing allogeneic hematopoietic stem cell transplantation. The analysis of patient/donor cell chimerism during the posttransplant period reveals immunological interactions between donor and recipient providing important information for preemptive therapeutic interventions. In addition to facilitating the assessment of successful engraftment, monitoring of chimerism at regular intervals can provide an early indication of imminent graft rejection[1–3] and, in patients with malignant hematological disorders, a timely alert of impending relapse.[4–8] The number of allogeneic stem cell transplants performed worldwide has been steadily growing over the past years. In particular, the increasing employment of reduced intensity conditioning regimens and cord blood transplants, which require very careful surveillance of the graft, has contributed to the clinical importance of chimerism testing. In contrast to fluorescence *in situ* hybridization, short tandem repeat (STR)-PCR can be applied to routine diagnostic monitoring of chimerism in any transplant setting. However, the heterogeneity of technical approaches to chimerism analysis and the diversity of STR markers used have compromised the comparison of results generated at different centers. The EuroChimerism (EUC) consortium (including 12 centers from 10 European countries; Table 1) has been constituted to address the urgent need for a standardized technology specifically adapted to the requirements of quantitative chimerism analysis. The paramount goal of the ensuing collaborative study termed EuroChimerism Concerted Action was to establish a standardized approach to quantitative chimerism testing with the aim to facilitate harmonization of chimerism diagnostics between European centers, and to provide a basis for appropriate quality control.

A variety of technical approaches to chimerism analysis are currently used. Techniques based on PCR amplification of polymorphic DNA sequences, facilitating unequivocal distinction and quantitative assessment of recipient- and donor-derived cells, have been the preferred approach to chimerism testing at most centers. Despite the potential of single nucleotide and insertion/deletion polymorphism analysis by real-time PCR,[4,9–16] the investigation of microsatellite (STR) polymorphisms is currently the most widely used technique in clinical chimerism testing.

[1]Children's Cancer Research Institute and Department of Pediatrics, Meduniwien, Vienna, Austria; [2]Department of Pediatrics, University Frankfurt, Frankfurt, Germany; [3]Department Transplantation Immunology Maastricht University, Maastricht, The Netherlands; [4]Department of Pathology University Medical Center, Utrecht, The Netherlands; [5]Hopital Robert Debré, Paris, France; [6]St James Hospital, Dublin, Ireland; [7]Department of Clinical and Biological Sciences, University Turin, Turin, Italy; [8]Department of Pediatrics, University Milano, Monza, Italy; [9]Department of Immunology, Erasmus University Rotterdam, Rotterdam, The Netherlands; [10]Department of Hematology, University Salamanca, Salamanca, Spain; [11]Clinical Genetics, University Hospital, Uppsala, Sweden; [12]Department of Internal Medicine, University Hospitals and University of Geneva, Geneva, Switzerland; [13]Royal Hospital for Children, Bristol, UK; [14]H&I Laboratory, National Blood and Transplant Service, Bristol, UK and [15]Department of Medical Statistics, CEMSIIS, Medical University Vienna, Vienna, Austria. Correspondence: Professor T Lion, Molecular Microbiology, Children's Cancer Research Institute and Department of Pediatrics, Meduniwien, Zimmermannplatz 10, Vienna A-1090, Austria.
E-mail: thomas.lion@ccri.at
Received 1 September 2011; revised 3 February 2012; accepted 29 February 2012; accepted article preview online 7 March 2012

Table 1. EuroChimerism consortium

Austria
Children's Cancer Research Institute, Vienna
Thomas Lion (Coordinator)
Franz Watzinger
Sandra Preuner
Department of Medical Statistics, CEMSIIS, Medical University Vienna
Florian Frommlet

Germany
Department of Pediatrics, University Frankfurt
Peter Bader (Deputy coordinator)
Hermann Kreyenberg

Italy
Department of Pediatrics, University Milano, Monza
Andrea Biondi
Federica Colnaghi
Department of Clinical and Biological Sciences, University Turin
Anna Serra
Giuseppe Saglio

Spain
Department of Hematology, University Salamanca
Marcos González-Díaz
Miguel Alcoceba

Switzerland
Department of Hematology, University Geneva
Eddy Roosnek
Solange Vischer

France
Hopital Robert Debré, Paris
Hélène Cavé
Cécile Acquaviva

Ireland
St. James Hospital, Dublin
Mark Lawler
Mireille Crampe
Karen Molloy

The Netherlands
Department of Transplantation Immunology Maastricht University Medical Center
Marcel Tilanus
Department of Pathology, University Medical Center, Utrecht
Roel de Weger
Joyce van Loon
Department of Immunology, Erasmus University Rotterdam
Jacques J.M van Dongen
Mirjam van der Burg

Sweden
Clinical Genetics, University Hospital, Uppsala
Gisela Barbany
Monica Hermanson

United Kingdom
Royal Hospital for Children, Bristol
Colin Steward
H&I Laboratory, National Blood and Transplant Service, Bristol
John Harvey

The methods of STR-PCR analysis performed in different diagnostic laboratories are usually based on rather diverse in-house assays, although attempts to introduce a commercially available platform for chimerism testing (http://www.biotype.de) or to exploit existing microsatellite kits designed for forensic purposes have been made. The EUC consortium has therefore established the basis for a diagnostic kit based on STR-PCR analysis, designed to optimally meet the requirements of clinical chimerism testing.

The EuroChimerism Concerted Action was supported by a grant from the European Commission (within the fifth Framework program) and included five work packages covering the most important aspects of chimerism analysis (Supplementary Table SI-1). In this report, we present the results of the EuroChimerism Concerted Action with the intention to provide an impetus for laboratories involved in chimerism testing to join this European initiative aiming at the establishment of widely accepted diagnostic standards. Based on the collaboration of the EUC consortium with an industrial partner (Miltenyi Biotech, Bergisch Gladbach, Germany) a multiplex PCR kit for the identification of informative STR markers (ChimerXplain, Bergisch-Gladbach, Germany) and a singleplex PCR kit specifically adapted for quantitative chimerism analysis (ChimerXact, Bergisch-Gladbach, Germany) will soon be commercially available, and may serve as a tool for the harmonization of posttransplant monitoring between diagnostic laboratories.

MATERIALS AND METHODS

Details of various methodological procedures are presented online as Supplementary Information, including the approaches to DNA isolation and appropriate controls,[17] PCR amplification of singleplex and multiplex reactions (Tables 2 and 3), capillary electrophoresis of amplicons, relevant definitions,[18] and detailed information on statistical analysis. Efficient approaches to DNA isolation for chimerism testing have been specifically addressed in a recent publication of the EUC consortium.[17]

Preparation of dilution series

As outlined in more detail in the statistics section (Supplementary Information), important parameters of chimerism analysis, including the detection limit (DL), reproducibility and accuracy were assessed by testing centrally prepared serial dilutions of recipient DNA in donor DNA mimicking different levels of mixed chimerism. Combinations of DNA samples providing informative allelic constellations were selected to prepare four different dilution series including the following steps: 50, 25, 12.5, 6, 3, 1.6 and 0.8%. In addition to the initial DNA measurement by optical density, the presence of identical concentrations of DNA in all steps of the dilution series was controlled by real-time PCR amplification of albumin as control gene.[19]

Quantitative analysis of chimerism status

Based on measurements of the donor/recipient peak height ratios, chimerism levels were calculated using formulas specifically adapted for different allelic constellations (Table 4).

Transfer of materials

At several stages of the study, different control materials including primers, DNA preparations and PCR products were centrally prepared and distributed either to a restricted number of laboratories or to all participating centers. In the present study, archived materials from

Table 2. EUC markers, primer sequences, fluorescence labels and primer concentrations in singleplex reactions

Marker	*Sequence (5′ – 3′)*	*Fluorescence label*	*Primer conc. (nM) for singleplex reactions*
D2S1360			
Fw	CTGCATTAAAACATTCGAAACCAA	JOE	80
Rev	GCAGCAGATTGTGGGACTTCTCAG		80
D7S1517			
Fw	AGCCTGATCATTACCAGGT	JOE	160
Rev	GTTTCTATTGGGGCCATCTTGC		160
D8S1132			
Fw	TCTCTCTCTCCCTCTCTCTTTCGAG	TMR	80
Rev	GTTTGCCATCTTCTTACCTCTGTTGGTC		80
D9S1118			
Fw	CAGGATATTATGTGATGGAATCC	FL	320
Rev	GATCTCTTCTCTCTCTCTCTTTCTCCC		80
D10S2325			
Fw	TATGGTGACCTTAAGCAGCCATG	JOE	80
Rev	GTGTCTTAGCTGAGAGATCACGCACTGC		80
D11S554			
Fw	GGTAGCAGAGCAAGACTGTC	FL	80
Rev	GTTTCACCTTCATCCTAAGGCAGC		80
D12S1064			
Fw	ACTACTCCAAGGTTCCAGCC	TMR	160
Rev	ACTGTTATCTCTCTTGTGGTAG		160
D12S391			
Fw	ATCAACAGGATCAATGGATGCAT	FL	160
Rev	GGGCTTTTAGACCTGGACTGAG		160
D17S1290			
Fw	CCAACAGAGCAAGACTGTC	FL	80
Rev	GTTTGAAACAGTTAAATGGCCAAAG		80
D19S253			
Fw	ATAGACAGACAGACGGACTG	FL	160
Rev	GTTTGGGAGTGGAGATTACCCCT		160
MYCL1			
Fw	AACCGTAGCCTGGCGAGACT	FL	160
Rev	GTTTCCTTTTAAGCTGCAACAAATTTC		160
P450CYP19			
Fw	GTTCCACATAATGAAGCACAATC	FL	240
Rev	GTTTAATCGCCTGAGTCCTGGGA		240
SE-33			
Fw	AATCTGGGCGACAAGAGTGA	FL	160
Rev	ACATCTCCCCTACCGCTATA		160

Abbreviations: EUC, EuroChimerism; Fw, forward; Rw, reverse. The acronyms JOE, TMR, and FL designate green, yellow, and blue fluorescence dyes, respectively.

Table 3. EUC multiplex panels

	Primer concentrations (nM)	*FL*	*JOE*	*TMR*
Group 1				
D9S1118	[a]	92 – 128		
MYCL1	240	156 – 225		
D7S1517	200		164 – 212	
D11S554	400			166 – 246
D8S1132	400			347 – 379
Group 2				
D10S2325	360	163 – 213		
D12S391	480	237 – 269		
P450CYP19	800	314 – 464		
D2S1360	800		233 – 273	

Abbreviation: EUC, EuroChimerism. The acronyms JOE, TMR, and FL designate green, yellow, and blue fluorescence dyes, respectively.
[a]D9S1118 asymmetric PCR: fw primer 640 nM and rev primer 160 nM. The range of allele sizes is indicated.

Table 4. Formulas for calculation of recipient chimerism levels for different recipient and donor allele constellations

Allele constellations	*Recipient chimerism level (%)*
RD, DR	$H_R/(H_R+H_D)$
RRD, RDR, DRR	$(H_{R1}+H_{R2})/(H_{R1}+H_{R2}+H_D)$
RDD, DRD, DDR	$H_R/(H_R+H_{D1}+H_{D2})$
RRDD, DDRR, DRRD, RDDR, RDRD, DRDR	$(H_{R1}+H_{R2})/(H_{R1}+H_{R2}+H_{D1}+H_{D2})$
RSD, DSR, SRD, SDR	$H_R/(H_R+H_D)$
rDR, RrD	$2*H_{R2}/(2*H_{R2}+H_D)$ $2*H_{R1}/(2*H_{R1}+H_D)$
RdrD	$H_{R1}/(H_{R1}+H_{D2})$
rdRD	$H_{R2}/(H_{R2}+H_{D2})$
RrDD	$2*H_{R1}/(2*H_{R2}+H_{D1}+H_{D2})$
rDRD	$2*H_{R2}/(2*H_{R2}+H_{D1}+H_{D2})$

The indicated examples of allele constellations are based on the RSD code (see Supplementary Figure 1). The formulas for calculation of chimerism levels are based on peak heights (H) of the recipient allele(s) (H_R, H_{R1}, H_{R2}) and the donor allele(s) (H_D, H_{D1}, H_{D2}), respectively. The subscripts indicate the shorter ($_1$) and the longer ($_2$) recipient ($_R$)/donor ($_D$) alleles, as present in the respective constellation. Alleles potentially affected by stutter peak formation
(lower case letters) are disregarded in the formulas for the calculation of chimerism, and are substituted by doubling the peak height of the unaffected allele.

patients who had given their consent to chimerism analysis before transplantation were used. Shipment was performed at ambient temperature by an overnight express service.

Statistical analysis

Definition of variables. To investigate the performance of the 13 markers of the EUC panel with regard to the DL and the accuracy of quantitative assessment of donor/recipient cell ratios, four dilution series including the dilution steps indicated above were generated. Based on the definitions of allelic constellations eligible for chimerism testing using the RSD code established by the EUC consortium,[18] a total of 36 informative marker constellations (IMCs) were available for investigation. The analysis was designed to address the question to which extent individual factors including the specific center involved, the dilution series used, the STR marker investigated and certain aspects of the IMC indicated below might have an influence on the two dependent variables-DL and divergence (accuracy).

The chimerism levels for different recipient and donor allele constellations, as specified by the RSD code,[18] were calculated according to the formulas indicated in Table 4 based on the respective donor and recipient peak heights. For IMCs potentially compromised by stutter peak formation, modified formulas were used in which the peak affected by the stutter was substituted by doubling the peak height of the unaffected recipient peak (Table 4).

Study design. The parameters including DL, reproducibility, divergence (accuracy) and linearity were determined by testing the dilution series of

recipient in donor DNA described above according to an incomplete balanced block design (Supplementary Information).[20]

Statistical methods. To analyze the influence of the factors center, dilution series and allelic imbalance on the DL, proportional odds models[21] using PROC LOGISTICS of SAS (SAS Institute Inc., 2000, Cary, NC, USA) were applied. The effects on accuracy of the random factors center, dilution series, marker, IMC, the fixed factors dilution step and the covariate allelic imbalance were investigated by a mixed model analysis using PROC MIXED of SAS[22] on the basis of reproducible data obtained from the participating centers (Supplementary Information).

RESULTS

Cell number and DNA template requirements for quantitative chimerism analysis

In comparison to the commonly performed chimerism testing within total white blood cells, assessment of chimerism within specific leukocyte fractions may provide earlier and more specific information on impending complications in various instances.[1,4,23] When rare cell subsets are isolated for subsequent chimerism analysis, the availability of sufficient amounts of DNA may become a limiting factor with regard to the achievable sensitivity and reproducibility of results. The experience among members of the EUC consortium indicated that the cell number available for analysis in these instances can be as low as a few hundred to a few thousand cells. Based on the DNA content of human nucleated cells and the efficiency of nucleic acid extraction, the amount of DNA available as template for subsequent chimerism analysis by PCR can therefore be as low as 1–10 ng. Based on this notion, we performed a series of experiments addressing the DL and the reproducibility of chimerism testing in samples containing 1 and 10 ng DNA template. Multiple dilution series representative of different clinical posttransplant specimens were tested in duplicate assays, focusing particularly on the analysis of samples containing 1 and 10 ng of total DNA template. The results indicated that DLs of 0.8–1.6% are achievable with either template amount in about 90% of instances. However, the reproducibility, defined as the concordance between replicate tests, was clearly superior in specimens containing 10 ng DNA template: the maximum differences observed in $\geqslant$90% of replicate measurements were $\pm$1% for the lowest dilutions containing 0.8–1.6% of donor or recipient DNA. These observations indicated that STR-PCR reactions containing 10 ng of template nucleic acid, corresponding to the DNA content of $\sim$1.500 human diploid cells, can be regarded as the minimum requirement for reproducible chimerism testing with adequate DLs. Based on these findings, all subsequent experiments were carried out using 10 ng of DNA per PCR reaction.

Establishment of the EUC STR marker panel

The EUC consortium has addressed the principles of marker eligibility for chimerism testing in a separate article in which the effect of allelic constellations on quantitative analysis is discussed, and the criteria for the selection of adequate microsatellite markers are presented.[18] The most important properties of eligible markers are outlined in the Supplementary Information.

The selection process of microsatellite loci optimally suited for quantitative chimerism testing was started by compiling the best performing markers from individual participating centers. This initial collection of markers, which is displayed in Table 5, was based on long-lasting experience with microsatellite analysis in the surveillance of chimerism in leading European laboratories. The main criteria for the compilation of the initial panel included the informativeness (see definitions in the Supplementary Information) and the DLs of individual markers observed in routine clinical diagnosis. As a first step of evaluation by the EUC, each of the markers from the initial panel was tested at different centers for its informativeness in a minimum of 50 related donor/recipient pairs (as related individuals display a particularly high probability of shared alleles). The best performing markers were selected for further testing, as described below and finally resulted in the establishment of the EUC STR marker panel (Table 2). Detailed analyses and allele frequencies of the 13 selected EUC STR markers are presented in Supplementary Tables SI-2a and b. Most of the markers display four-nucleotide repeat motifs, but some contain combinations of different tetranucleotide repeat sequences.[24] The predominant variable repeats are indicated in Table 5, and details of the complex repeats were described earlier.[24] Upon establishment of the EUC marker panel, a variety of different primers, combinations and concentrations were tested for each individual marker. The criteria of eligibility included (a) reproducible achievement of peak heights $\geqslant$6.000 RFU with 10 ng DNA template, (b) secondary stutter peaks $<$1% of main peaks and (c) absent or low background noise ($<$50 RFU). The selected primers and combinations were optimized for quantitative analysis of chimerism under uniform amplification conditions (Supplementary information).

Table 5. Primary panel of STR markers investigated

Name	*Repeat*	*Motif*
D12S391	Tetra	$[GATA]_n$ $[GACA]_n$
D20S194	Di	$[CA]_n$
D12S1064	Tetra	$[TATC]_n$
D17S1290	complex	$[TGA]_n[TAGA]_n$
D13S317	Tetra	$[TATC]_n$
CSF1PO	Tetra	$[AGAT]_n$
MYCL1	complex[a]	$[GAAA]_n[GAAAA]_n$
P-450 Cyp19	Tetra/Penta	$[TATTT]_n[TATT]_n$
vWF II	Tetra	$[TCTA]_n$
D3S1768	Tetra	$[TAGA]_n$
D9S1118	Tetra	$[TATC]_n$
D11S554	complex	$[AG]_n$ $[AAAG]_n$
D19S253	Tetra	$[ATAG]_n$
D7S1517	Tetra	$[GAAA]_n[CAAA]_n$
D10S2325	Penta	$[TCTTA]_n$
D2S1360	Tetra	$[TAGA]_n[CAGA]_n$
D8S1132	Tetra	$[CTAT]_n$
SE33	complex	$[AAAG]_n$

[a]The predominant repeat motifs of the complex repeat markers are indicated. The most variable motifs of the tetranucleotide repeats are indicated. Details of the repeat content has been described elsewhere.[22]

Informativeness of the EUC STR marker panel

The informativeness of individual microsatellite loci was determined by testing their ability to provide allelic constellations eligible for quantitative chimerism analysis according to the guidelines of the EUC.[18] Briefly, the minimum requirements for an eligible allelic constellation include the presence of at least one unique donor and recipient allele and a distance between informative alleles corresponding to at least two tandem repeat units. The informativeness of markers included in the initial panel was determined under stringent conditions reflecting the most challenging situation, which is the related transplant setting. In total, 740 related and 159 unrelated donor/recipient pairs were tested by the EUC marker panel. EUC markers were ranked according to the previously defined criteria.[18] For all markers, the heterozygosity index, the polymorphism information content (PIC value)[25] and the combined probability were calculated (Table 6).

The informativeness of the EUC panel was compared with that of a commercial multiplex microsatellite marker kit designed for forensic analysis (Powerplex16, Promega, Mannheim, Germany).

Table 6. Informativeness of the STR markers

EUC markers				
Name	*HI*	*PIC value*	*EUC criteria*	*Combined probability*
SE33	0.9412	0.9380	0.8277	0.8277
D11S554	0.9424	0.9396	0.7115	0.9503
D9S1118	0.8412	0.8221	0.5447	0.9774
D7S1517	0.8630	0.8492	0.4945	0.9886
D2S1360	0.8459	0.8324	0.4915	0.9942
D10S2325	0.8728	0.8595	0.4782	0.9970
MYCL-1	0.9282	0.9242	0.4614	0.9984
D12S391	0.8748	0.8629	0.4583	0.9991
D17S1290	0.8459	0.8282	0.4059	0.9995
D8S1132	0.8641	0.8487	0.3959	0.9997
D19S253	0.7982	0.7687	0.3706	0.9998
D12S1064	0.8485	0.8307	0.3331	0.9999
P450-CYP19	0.7649	0.7252	0.2850	0.9999

The Heterozygosity Index (HI) and Polymorphic information content (PIC) values were calculated from the allele frequencies for each EUC-STR (EuroChimerism-short tandem repeat) marker. The frequencies considering the EUC criteria reflect the informativeness based on the likelihood to identify patient- and donor-specific allele(s) separated by at least two repeat lengths (to prevent any influence of stuttering on the peak height). The combined probability (CP) indicates the increasing chance of finding informative markers by testing additional microsatellites. Under the stringent EUC criteria, analysis of five EUC markers provides a 99% chance of finding one or more informative markers in any patient/donor constellation.

The kit, which includes 16 microsatellite loci, was tested in 320 related donor/recipient pairs and revealed a level of informativeness inferior to the EUC panel, with regard to the specific needs of chimerism analysis.

The standards established by the EUC consortium requesting the availability of at least two bidirectionally informative microsatellite markers for chimerism testing in any patient/donor constellation was met by the EUC panel. Combined analysis of the five most informative STR markers including SE33, D11S554, D9S1118, D7S1517 and D2S1360 revealed a $>99\%$ probability of finding bidirectionally informative allelic constellations, a level that could not be achieved with the Powerplex16 kit. The EUC panel revealed multiple markers eligible for chimerism testing in virtually all instances, even under the stringent conditions of the related donor/recipient setting. The high level of informativeness for chimerism testing provided by the loci included in the EUC panel is documented by the allele frequencies of individual markers (Supplementary Tables SI-2a and b).

Multiplexing of EUC markers

The monitoring of chimerism by STR-PCR is most commonly performed by assays based on the amplification of a single microsatellite marker per reaction, because singleplex PCR assays generally provide the highest achievable level of sensitivity. For the initial selection of one or more eligible markers, however, it is advantageous to employ a multiplex test facilitating simultaneous testing of multiple microsatellite loci. Markers that can be tested in a multiplex reaction either must have a non-overlapping spectrum of allele sizes or the PCR products of each marker must be labelled differentially to permit clear assignment of the amplification products by fluorescence-based electrophoresis. A number of fluorescent dyes are available for the labelling of amplification primers, thereby permitting coamplification of multiple microsatellite targets. There are, nevertheless, limitations with regard to the number and type of microsatellite loci that can be compiled in a multiplex PCR reaction. Owing to the high level of informativeness of the markers included in the EUC panel, only a limited number of loci need to be tested in a multiplex assay to identify more than one marker eligible for quantitative monitoring of chimerism in any donor/recipient constellation. It appeared reasonable therefore to establish multiplex assays targeting a restricted number of microsatellite loci. Based on the number of currently available dyes and the allelic spectra of the EUC markers, two multiplex assays, one pentaplex and one tetraplex, were established. The loci included in the multiplex reactions and the respective primer concentrations are indicated in Table 3. The probability of identifying more than one informative marker in any given donor/recipient pair was calculated for both multiplex reactions on the basis of allelic frequencies in the European population covering patients with diverse ethnic backgrounds: under the stringent criteria of eligibility, the probability is 84.5% for the pentaplex assay, 57.6% for the tetraplex assay and 97.7% for both assays combined. These numbers indicate that initial screening by the pentaplex assay alone will be sufficient to provide at least two eligible markers for posttransplant chimerism testing in most instances. Concomitant employment of both multiplex assays, or implementation of additional markers from the EUC panel currently not included in any of the multiplex reactions, will only be required in rare instances. It is conceivable, however, that the current multiplex assays will be further optimized by the manufacturer of the diagnostic kit (Miltenyi Biotec; see below).

DL of the EUC markers

Statistical analysis of DLs (i.e., sensitivity) and other validation parameters of the EUC panel were based on an incomplete balanced block design (see Materials and Methods, statistical analysis-Supplementary Information). The DL for the 13 markers of the EUC panel was between 0.8 and 1.6% in the great majority of the samples analyzed. The lowest limit of detection tested (0.8%) was reached in 66% of all analyses, whereas 22.3% of the tests had a DL between 0.8 and 1.6%, and 10.4% of tests between 1.6 and 3%. In three instances only (1.4%), the DL achieved was between 3 and 6%. At first sight, it appeared intriguing that the analysis of three STR markers (D17S1290, D12S1064 and D19S25) revealed a DL of 0.8% in all experiments performed. This observation, however, was shown to be attributable to particular allelic constellations rather than to the markers themselves. Analysis of the DLs in relation to the allelic constellation revealed that in cases displaying homozygous recipient and heterozygous donor alleles, a DL of 0.8% for recipient cells was reached in most instances (93%), and a limit of 1.6% in the remaining tests. By contrast, the few analyses displaying a DL for recipient cells of $\geqslant 3\%$ had the opposite allelic constellation (recipient heterozygous/donor homozygous). Our observations indicate that this constellation tends to reveal lower recipient peaks, which sometimes remain below the minimum requirement of unequivocally detectable signals (<50 RFU) even at recipient chimerism levels $\geqslant 3\%$, despite the employment of optimized detection parameters. In contrast to the allelic constellation, logistic regression analysis of other factors including allelic imbalance, dilution series or center revealed no influence on the achievable DLs.

Reproducibility and accuracy of quantitative analysis

Analysis of the factors reproducibility and accuracy was also based on the incomplete balanced block design. The concordance between replicate singleplex assays based on the analysis of 10 ng of template DNA revealed a maximum intrinsic variability of $\pm 1\%$ and $\pm 2-3\%$ for samples with low ($\leqslant 1.6\%$) and higher proportions ($>3\%$) of recipient cells, respectively. The parameter accuracy, which displayed a somewhat higher degree of intrinsic variability, was therefore identified as a more relevant determinant

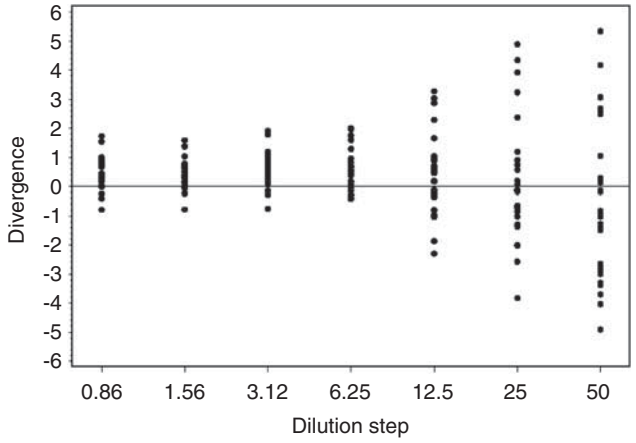

Figure 1. EUC markers—accuracy of quantitative assessment. The raw data of the assessment of accuracy indicated by the divergence (in percent; *y* axis) between measured (individual dots) and true values (=dilution steps; *x* axis) are displayed for the analyses performed.

for quantitative analysis of chimerism and the establishment of appropriate confidence intervals. The raw data reflecting the accuracy of measurements, assessed by the disparity between the actual recipient/donor ratio in defined cell mixtures and the experimentally determined values of chimerism (Figure 1), indicated the greatest deviations of up to ±5% in 50/50 mixtures of recipient in donor DNA. Deviations from ideal values observed in mixtures containing lower proportions of recipient DNA (25–12.5%) were less pronounced, as shown in Figure 1. Mixtures containing percentages of recipient DNA ranging between 0.8 and 6% showed a very similar pattern of divergence from the ideal values, with a tendency to overestimate the level of chimerism by up to about 2% (Figure 1).

Impact of allelic constellation and unbalanced amplification on quantitative analysis

The divergence between the true and the experimentally determined chimerism levels was assessed by testing well-defined serial dilutions of recipient in donor DNA according to the mixed model analysis described in the statistical methods section. Although certain variables including the individual centers and dilution series showed no significant effect of the divergence, a differential influence of the microsatellite marker used was observed. Detailed analysis revealed, however, that the effect on divergence was not attributable to the marker itself ($P=0.35$), but rather to the specific allelic constellation (IMC; $P=0.006$), which was an important variable (random factor) in the final mixed model analysis.

Moreover, the factor allelic imbalance was identified as an important variable significantly affecting the accuracy of quantitative analysis of chimerism ($P=0.02$): in principle, longer DNA fragments tend to be amplified less efficiently by PCR. Although the difference in size between sister alleles of judiciously selected STR markers is generally small, preferential amplification of shorter alleles leading to higher peaks has been observed in heterozygous constellations. This phenomenon, referred to as allelic imbalance, did not show any influence on the DL, but can affect quantitative analysis of STR-PCR assays in terms of accuracy. For markers of the EUC panel, the maximum bias attributable to this phenomenon, in the range of ±2%, was observed in the presence of large distances between informative donor and recipient alleles (>40 bp), and mixed chimerism levels at 50%. The maximum bias was less pronounced at all other levels of mixed chimerism. For levels of donor or recipient chimerism below 6%, the maximum bias conferred by allelic imbalance was only in the range of ±0.25%. The experience of the EUC consortium indicated that the occurrence of unbalanced allele amplification was largely attributable to suboptimal set-up of PCR assays. The factors influencing the balance between amplification efficiencies of STR sister alleles were shown to include primarily the amount of DNA template and the total number of PCR cycles performed. As sister alleles do not have the properties of perfect competitors in the reaction, imbalances may occur particularly if the PCR is performed beyond the exponential phase of amplification. In the presence of 10 ng of template DNA, which was the standard amount in all singleplex assays using the EUC marker panel, amplification under the conditions indicated in Materials and Methods was shown to prevent relevant inaccuracies of quantitative analysis in heterozygous allelic constellations.

Table 7. Confidence intervals of accuracy defined for recipient chimerism results

Percentage recipient chimerism	*Posttransplant monitoring*			
	By a single STR marker		*By two or more STR markers*	
	CI (95%)	*CI (99%)*	*CI (95%)*	*CI (99%)*
< 6% or >94%	m ± 1.6	m ± 2.1	m ± 1.8	m ± 2.4
6–12% or 87–94%	m ± 2.8	m ± 3.7	m ± 3.3	m ± 4.3
12–25% or 75–87%	m ± 3.0	m ± 4.0	m ± 4.3	m ± 5.7
25–75%	m ± 3.8	m ± 5.0	m ± 5.7	m ± 7.5

The pointwise two-sided confidence intervals (CI) are based on a mixed-effects model including the random factor IMC (informative marker constellation), and the fixed factor allelic imbalance. The 95 and 99% CI on the left are valid for any allelic constellation occurring in a given EUC (EuroChimerism) marker, and are applicable if the monitoring of chimerism in a transplant recipient is performed by a single STR (short tandem repeat) marker. In instances in which a patient is monitored by two or more different STR markers, the CI values on the right, which account for the variation between different marker constellations, need to be applied. Measured values (m) in consecutive patient samples which lie within the indicated CI can be regarded as intrinsic variability of the method. Conversely, consecutive values outside the CI reliably reflect actual changes in the level of chimerism.

Approach to calculation of recipient/donor chimerism

The heights and peak areas of fluorescence signals provided by capillary electrophoresis were evaluated by analyzing a large series of PCR amplification products generated by EUC STR markers. Quantitative assessment of chimerism based on either heights or areas of individual peaks yielded comparable results. In view of the more convenient determination, peak heights were used for all subsequent analyses of chimerism throughout the study. Based on the background noise, the lower threshold for signals that can be regarded as positive was set at 50 RFU. For quantitative analysis of minor signals, the peak heights of dominant alleles must not be off-scale (the maximal legitimate height depends on the instrument used). Conversely, the peaks must display a defined minimal height (minimal threshold of peak heights; MTPH) in order to permit the detection of minor signals with adequate sensitivity, as outlined in the Materials and Methods section. The above prerequisites for the calculation of chimerism were achieved by appropriate adjustment of analysis parameters for capillary electrophoresis including the injection time and voltage, and if required, the amount of PCR product analyzed. When the indicated criteria for quantitative analysis were met, calculation of the chimerism levels was performed by

implementing arithmetic formulas accounting for different allelic constellations (Table 4).

Interpretation of chimerism levels

Changes in the level of chimerism can be interpreted as actual increases or decreases, if the measured values of consecutive samples are not within the intrinsic variability of the assay. Hence, calculation of pointwise two-sided 95 and 99% confidence intervals (Table 7) was performed for the assessment of individual recipient/donor cell proportions, in order to provide a basis for reliable quantitative analysis and monitoring of chimerism. The EUC consortium has defined differences between subsequent measurements as real changes in the level of chimerism if the values were outside the 95% or more preferably the 99% confidence intervals.

DISCUSSION

In this report, we present the results of the EuroChimerism Concerted Action, which aimed at the establishment of a standardized, European-harmonized approach to the detection and monitoring of recipient/donor chimerism after allogeneic stem cell transplantation. The availability of an assay optimized for chimerism analysis in the European population should help overcome the disadvantages resulting from the great heterogeneity of tests currently used for posttransplant monitoring. Currently, nucleic acid amplification procedures based on in-house assays or commercial kits for multiplex analysis of microsatellite (STR) loci designed for forensic purposes are commonly being used. The latter certainly provide a potentially useful option for standardized analysis of chimerism, and the EUC consortium has evaluated the adequacy of a commercial multiplex kit comprising 16 microsatellite markers (PowerPlex 16, Promega) for this purpose. However, the requirements for the eligibility of microsatellite markers for clinical testing of chimerism are far more stringent than those for forensic analysis. The allelic constellations eligible for application in chimerism testing require not only clearly distinguishable allelic patterns of donor and recipient, but also a number of additional features relevant for quantitative analysis of allele ratios.[18] Essential criteria for marker selection established by the EUC consortium include adequate achievable sensitivity and the presence of bidirectionally informative recipient and donor alleles unaffected by stutter peak formation as a prerequisite for accurate quantitative analysis.

As specified below, our aim was the availability of at least two alternative, informative markers eligible for quantitative monitoring of chimerism according to the quality standards of the EUC consortium in any donor/recipient constellation, which may be a particular challenge in the related transplant setting. With regard to the presence of multiple informative markers, testing of the forensic multiplex kit in a large series of donor/recipient pairs revealed suboptimal results, thus emphasizing the need for a marker panel optimized for the specific requirements of chimerism testing. The employment of multiplex reactions is instrumental in identifying markers suitable for subsequent monitoring of chimerism, but our observations indicated that large multiplex assays often do not provide the desired level of sensitivity necessary for the assessment of lower levels of chimerism. This limitation is attributable to the high degree of complexity of multiplex reactions, and is apparently an inherent property of assays targeting multiple STR markers in individual PCR reactions (http://www.promega.com/applications; https://products.appliedbiosystems.com; http://www.biotype.de). We have therefore established a panel of judiciously selected microsatellite markers with optimized primer combinations permitting PCR amplification under uniform conditions. The standardized PCR protocol facilitates both the performance of multiplex reactions for rapid selection of informative markers, and singleplex reactions for optimal sensitivity and quantitative analysis of recipient- and donor-derived cells. The EUC panel was established on the basis of a two-step selection process. First, a primary microsatellite panel was complied by collecting the best-performing markers used at the participating centers (Table 5). Subsequently, the pre-selected markers were subjected to detailed analysis of allele frequencies covering a broad spectrum of ethnic backgrounds in the European population by testing a large series of related and unrelated donor/recipient pairs in all centers of the consortium. For each microsatellite marker tested, an important prerequisite for passing this step of selection was the frequency of alleles complying with the requirements for quantitative analysis of chimerism, as defined by the consortium.[18] On the basis of detailed investigation of allelic frequencies, the EUC panel was demonstrated to provide multiple informative markers for the monitoring of chimerism in any donor/recipient constellation, and was shown to be superior in this regard to the commercial microsatellite panel developed for forensic purposes. In contrast to this kit, the higher degree of informativeness of the EUC panel can be expected to provide a selection of markers optimally suited for quantitative monitoring of chimerism according to the stringent criteria established by our consortium[18] in virtually any instance. The general availability of multiple STR loci eligible for subsequent chimerism testing offers the possibility to monitor a patient by two or more different markers providing additional controls for quantitative assessment of chimerism. The comparison of independent measurements of chimerism or the calculation of the mean of different measurements may be desirable in certain situations to ensure the highest possible accuracy of quantitative chimerism analysis.

Owing to the uniform conditions of amplification for all markers included in the EUC panel, it is also conceivable to coamplify two or three markers in one reaction without having to perform extensive modifications of the assay. In this way, the level of chimerism could be assessed as a median value of the markers included, and the limited complexity of the reaction may be expected to exert a minor effect on the DL of the assay.

The strength of the EUC panel is, however, only partly attributable to the composition of markers and their high level of informativeness. More importantly, the establishment of singleplex assays with optimized primers and reaction conditions for all markers of the EUC panel provided the basis for the evaluation of essential parameters for standardized monitoring of chimerism including sensitivity, reproducibility and accuracy, the latter being a prerequisite for reliable assessment of quantitative changes between consecutive samples. Singleplex reactions for the analysis of individual markers of the EUC panel were shown to have a DL for minor cell populations in the range of 0.8–1.6% of in most instances, and to display an excellent reproducibility in the presence of adequate amounts of DNA template. In our study, PCR reactions containing 10 ng of template, which corresponds to the DNA content of about 1.500 diploid cells, were demonstrated to provide the desired levels of reproducibility and sensitivity. The use of lower DNA template amounts, as recommended in the commercial multiplex kit tested, was associated with lower achievable levels of sensitivity.

With regard to accuracy, the EUC panel is, to the best of our knowledge, the only STR-PCR-based assay for chimerism testing available in which the confidence intervals for quantitative analysis have been established. The availability of this information for all EUC singleplex PCR assays permits clear discrimination between values representing actual changes in the level of chimerism, and values that may indicate intrinsic variations inherent in the methodology. This aspect is of particular relevance for early assessment of rising recipient chimerism, which may facilitate timely prediction of impending graft rejection or relapse.[1–8]

Taken together, thorough investigation of the EUC panel in the European population and detailed analysis of individual markers demonstrated that the test system very adequately meets the specific requirements of quantitative analysis of chimerism, and provides an attractive option for diagnostic monitoring of patients during the posttransplant period. The availability of a standardized and European-harmonized methodology for chimerism testing is an important step towards optimal surveillance and management of allogeneic stem cell transplant recipients.

Concluding remarks

The EUC consortium has selected an industrial partner, Miltenyi Biotec, to integrate the EUC marker panel (patent pending) into a ready-to-use kit for chimerism testing. The availability of a commercial kit produced under controlled industrial conditions should promote wide implementation of a standardized methodology for the detection (ChimerXplain) and quantitative monitoring (ChimerXact) of chimerism in the clinical setting. Broad use of the off-the-shelf kit could help eliminate the problems of heterogeneity in the technical approaches used, and would greatly facilitate the comparison of data and the exchange of information between centers.

CONFLICT OF INTEREST

The EUC marker panel has been filed for patenting (A948/2006; PCT/AT2007/000266; EP2 024 508-2009) and the IP rights were assigned to the EuroChimerism consortium by an official document (Consortial Agreement). The IP rights were subsequently obtained by the industrial partner of the EuroChimerism consortium, Milenyi Biotec, Bergisch Gladbach, Germany. Revenues resulting from the recently launched kit based on the EUC panel must be used for further activities of the EuroChimerism Consortium in line with regulations specified in the consortial agreement.

ACKNOWLEDGEMENTS

We are grateful to the EU officers who have supervised and supported the project, including particularly Jaime Renart, Cornelius Schmaltz, Maria-José Vidal-Ragout and Micheline Divaris-Lambert. Moreover, we greatly appreciate the financial administrative help of Claudia Hochweis, Stefan Grünert, Alexandra Lidy and Karla Valdés-Rodriguez, the secretarial support of Ingrid Kerschberger and Nicole Reisinger at the coordinating center (CCRI) and the contributions of numerous technicians at the participating centers. The EuroChimerism Concerted Action (full project title: Diagnostic approaches to chimerism testing after allogeneic stem cell transplantation for early detection of graft rejection and relapse: technical development, standardization and European-coordinated clinical implementation) was carried out with financial support from the European commission within the call Quality of Life and Management of Living Resources, Fifth Framework Program, project No QLG1-CT-2002-01485.

REFERENCES

1 Matthes-Martin S, Lion T, Haas OA, Frommlet F, Daxberger H, Konig M *et al.* Lineage-specific chimaerism after stem cell transplantation in children following reduced intensity conditioning: potential predictive value of NK cell chimaerism for late graft rejection. *Leukemia* 2003; **17**: 1934 – 1942.
2 Liesveld JL, Rothberg PG. Mixed chimerism in SCT: conflict or peaceful coexistence? *Bone marrow transplant* 2008; **42**: 297 – 310.
3 Lawler M, McCann SR, Marsh JC, Ljungman P, Hows J, Vandenberghe E *et al.* Serial chimerism analyses indicate that mixed haemopoietic chimerism influences the probability of graft rejection and disease recurrence following allogeneic stem cell transplantation (SCT) for severe aplastic anaemia (SAA): indication for routine assessment of chimerism post SCT for SAA. *Br J Haematol* 2009; **144**: 933 – 945.
4 Lion T, Daxberger H, Dubovsky J, Filipcik P, Fritsch G, Printz D *et al.* Analysis of chimerism within specific leukocyte subsets for detection of residual or recurrent leukemia in pediatric patients after allogeneic stem cell transplantation. *Leukemia* 2001; **15**: 307 – 310.
5 Bader P, Kreyenberg H, Hoelle W, Dueckers G, Kremens B, Dilloo D *et al.* Increasing mixed chimerism defines a high-risk group of childhood acute myelogenous leukemia patients after allogeneic stem cell transplantation where pre-emptive immunotherapy may be effective. *Bone marrow transplant* 2004; **33**: 815 – 821.
6 Bader P, Kreyenberg H, Hoelle W, Dueckers G, Handgretinger R, Lang P *et al.* Increasing mixed chimerism is an important prognostic factor for unfavorable outcome in children with acute lymphoblastic leukemia after allogeneic stem-cell transplantation: possible role for pre-emptive immunotherapy? *J Clin Oncol* 2004; **22**: 1696 – 1705.
7 Bader P, Duckers G, Kreyenberg H, Hoelle W, Kerst G, Lang P *et al.* Monitoring of donor cell chimerism for the detection of relapse and early immunotherapeutic intervention in acute lymphoblastic leukemias. *Ann Hematol* 2002; **81** (Suppl 2): S25 – S27.
8 Bader P, Niemeyer C, Willasch A, Kreyenberg H, Strahm B, Kremens B *et al.* Children with myelodysplastic syndrome (MDS) and increasing mixed chimaerism after allogeneic stem cell transplantation have a poor outcome which can be improved by pre-emptive immunotherapy. *Br J Haematol* 2005; **128**: 649 – 658.
9 Thiede C, Bornhauser M, Oelschlagel U, Brendel C, Leo R, Daxberger H *et al.* Sequential monitoring of chimerism and detection of minimal residual disease after allogeneic blood stem cell transplantation (BSCT) using multiplex PCR amplification of short tandem repeat-markers. *Leukemia* 2001; **15**: 293 – 302.
10 Lion T. Summary: reports on quantitative analysis of chimerism after allogeneic stem cell transplantation by PCR amplification of microsatellite markers and capillary electrophoresis with fluorescence detection. *Leukemia* 2003; **17**: 252 – 254.
11 Kreyenberg H, Holle W, Mohrle 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 – 240.
12 Acquaviva C, Duval M, Mirebeau D, Bertin R, Cave H. Quantitative analysis of chimerism after allogeneic stem cell transplantation by PCR amplification of microsatellite markers and capillary electrophoresis with fluorescence detection: the Paris-Robert Debre experience. *Leukemia* 2003; **17**: 241 – 246.
13 Hancock JP, Goulden NJ, Oakhill A, Steward CG. Quantitative analysis of chimerism after allogeneic bone marrow transplantation using immunomagnetic selection and fluorescent microsatellite PCR. *Leukemia* 2003; **17**: 247 – 251.
14 Chalandon Y, Vischer S, Helg C, Chapuis B, Roosnek E. Quantitative analysis of chimerism after allogeneic stem cell transplantation by PCR amplification of microsatellite markers and capillary electrophoresis with fluorescence detection: the Geneva experience. *Leukemia* 2003; **17**: 228 – 231.
15 Thiede C. Diagnostic chimerism analysis after allogeneic stem cell transplantation: new methods and markers. *Am J Pharmacogenomics* 2004; **4**: 177 – 187.
16 McCann SR, Crampe M, Molloy K, Lawler M. Hemopoietic chimerism following stem cell transplantation. *Transfus Apher Sci* 2005; **32**: 55 – 61.
17 van der Burg M, Kreyenberg H, Willasch A, Barendregt BH, Preuner S, Watzinger F *et al.* Standardization of DNA isolation from low cell numbers for chimerism analysis by PCR of short tandem repeats. *Leukemia* 2011; **25**: 1467–1470.
18 Watzinger F, Lion T, Steward C. 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 – 1452.
19 Pongers-Willemse MJ, Verhagen OJ, Tibbe GJ, Wijkhuijs AJ, de Haas V, Roovers E *et al.* Real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia using junctional region specific TaqMan probes. *Leukemia* 1998; **12**: 2006 – 2014.
20 John A, Quenouille M. *Experiments: Design and Analysis* 2nd edn. Macmillan Publisher: New York, 1977.
21 McCullagh P. Regression models for original data. *J Roy Statist Soc B* 1980; **42**: 109 – 127.
22 Cary N. *SAS OnlineDoc, Version 8*. In: Cary N, editor. SAS Institute Inc., 2000.
23 Breuer S, Preuner S, Fritsch G, Daxberger H, Koenig M, Poetschger U *et al.* Early recipient chimerism testing in the T- and NK-cell lineages for risk assessment of graft rejection in pediatric patients undergoing allogeneic stem cell transplantation. *Leukemia* 2012; **26**: 509 – 519.
24 Tilanus MG. Short tandem repeat markers in diagnostics: what's in a repeat? *Leukemia* 2006; **20**: 1353 – 1355.
25 Guo X, Elston RC. Linkage information content of polymorphic genetic markers. *Hum Hered* 1999; **49**: 112 – 118.

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)