Establishing the method of chimerism monitoring after allogeneic stem cell transplantation using multiplex polymerase chain reaction amplification of short tandem repeat markers and Amelogenin

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We describe the implementation, optimization, sensitivity determination and first clinical results of polymerase chain reaction (PCR) amplification of polymorphic short tandem repeat (STR) markers and Amelogenin locus coupled with fluorescent detection and capillary electrophoresis in chimerism monitoring of patients transplanted at three different transplant centers using a commercially available multiplex microsatellite assay. The chimerism analysis was performed with genomic DNA extracted from unselected peripheral blood leukocytes of one hundred pediatric and adult patients, who underwent allogeneic stem cell transplantation (SCT) from human leukocyte antigen (HLA) matched or one antigen mismatched related or unrelated donors for malignant (70 patients) and non-malignant (30 patients) diseases. Tested were 79 donor recipient pairs for 15 STR systems and identified an informative marker in all but one of them (98,7%), using 6 selected systems out of these fifteen, that appeared highly informative in our patients' population. In 21 sex-mismatched donor recipient pairs we used the Amelogenin locus to distinguish the X and Y chromosome. In sixty-three out of these 100 patients chimerism was regularly analyzed from blood samples taken at various time points after SCT with the median follow up of 17 months. Complete chimerism (CC), maintained over the whole follow-up period, was detected in 24 (38, 1%), stable and decreasing mixed chimerism (MC) in 28 (44, 4%) and increasing MC in 11 patients (17, 5%). Patients with CC, stable and decreasing MC showed a significantly better (p 0,005) overall survival rate (0, 81), compared to those with increasing MC (0, 24). These results demonstrate that STR-based chimerism monitoring with sensitivity above 1% and high informativity (98, 7% of donor recipient pairs) is necessary in establishing the origin of engrafted cells after an allogeneic SCT, in detecting graft rejection and that it may contribute in identifying patients with imminent leukemia relapse.

Key words: allogeneic stem cell transplantation, chimerism monitoring, polymerase chain reaction, short tandem repeat markers, complete chimerism, decreasing mixed chimerism, increasing mixed chimerism

Over the past decades, allogeneic SCT has gained increasing importance as a treatment option for both malignant and non-malignant disorders in adult as well as pediatric patients [1, 2]. Considerable progress has been made in the analysis of hematopoietic chimerism afterwards and the molecular monitoring of the genotypic origin of engrafted cells has become a routine diagnostic tool to document engraftment and to detect graft rejection or impending relapse, at most centers performing allogeneic SCT [3-7]. The term "chimerism" was introduced in the field of medicine by Anderson et al [8] to describe organisms whose cells derive from two or more zygote lineages.

Close surveillance of chimerism within total peripheral blood leukocytes after an allogeneic SCT seems an indispensable tool for the clinical management of transplant recipients [9]. Therefore it is mandatory to use a highly sensitive, informative and accurate method of chimerism quantification. One of the most widespread methods of chimerism analysis is the PCR-based

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analysis of highly polymorphic STR markers revealed by capillary electrophoresis with the use of fluorescently labeled PCR primers that make the direct quantification of donor and recipient DNA possible on an automated DNA sequencer [4, 10-14]. More recently commercially available STR multiplex amplification kits are used because they owe a high degree of standardization and informativity [14-17].

Here, we report the implementation of this method of chimerism monitoring, its sensitivity and accuracy determination, as well as the first clinical results obtained at three Slovak bone marrow transplantation centers.

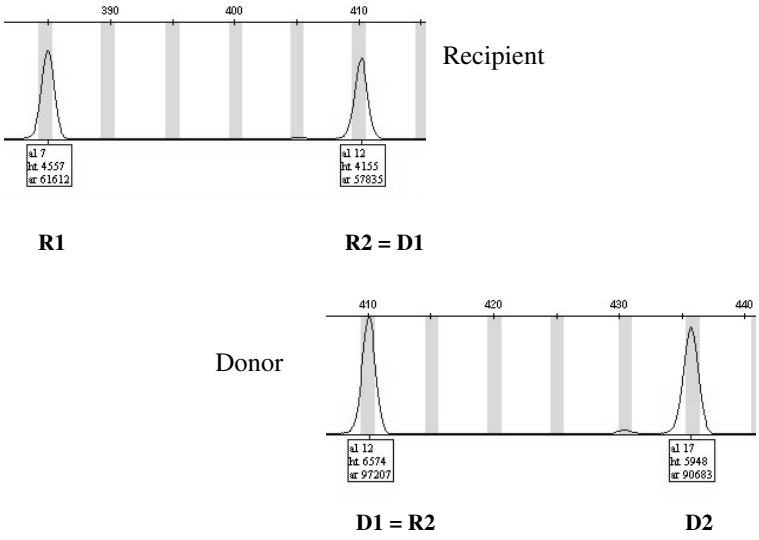

Figure 1. Informative pentanucleotide STR marker Penta E (recipient and donor are sharing allele 12)

Patients and methods

Patients. In one hundred patients from two adult and one pediatric transplantation centers who underwent allogeneic SCT for malignant (70 cases) and non-malignant (30 cases) diseases, chimerism analysis of polymorphic STR markers was performed. Out of these one hundred patients 37 were excluded from this study because they lacked early post-transplant blood samples for analysis. We further evaluated a cohort of 63 patients (33 males and 30 females) with the median age of 27 years (range 1 to 58) transplanted between June 2003 and October 2006. They received an allogeneic SCT from a HLA matched or one antigen mismatched related or unrelated donor. Majority of the patients were transplanted for malignant diseases (76, 2%) and received peripheral blood stem cells (87, 3%) as the stem cell source. Written informed consent was obtained from the patients or patients´ parents, in case of children, according to the institutional guidelines. The main characteristics of the studied cohort are summarized in Table 1.

Sample collection. Whole peripheral blood samples were collected for DNA extraction from both the donor and recipient before transplantation in order to determine an informative STR marker. The samples were collected at weekly or monthly intervals during the first 100 days, and monthly or every 2-3 month thereafter during the first year according to the transplantation center. During the second year the frequency was reduced to twice a year, only if the clinical situation warranted, more frequent chimerism analyses were performed.

DNA extraction. Genomic DNA was extracted from 200 µl of fresh or frozen peripheral blood using a column-based DNA isolation technique (Qiagene DNA Blood mini kit, QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA quantification was performed using standard UV absorption at 260 nm and DNA samples were stored until use at –80°C.

Artificial mixed chimeric samples preparation. In order to simulate different clinical situations post-transplant artificial mixtures of whole peripheral blood and DNA from healthy unrelated male and female individuals in different proportions were prepared. In this experiment one of the individuals was considered as the recipient and one as the donor of a fictitious SCT and they were chosen for this study after initial STR evaluation, which showed non-shared, informative STR alleles. For sensitivity and accuracy calculation, following serial mixtures of peripheral blood and DNA were applied: 100, 75, 50, 25, 5, 1 and 0, 1%. Sensitivity testing was performed for

Table 1. Patient characteristics

Characteristic	*Number (%)*
Patients/Transplants	63/66
Median age (years) (range)	27 (1-58)
Sex (male/female)	33/30
Diagnoses	
Malignant diseases	44 (76, 2%)
ALL	16
AML	22
NHL	3
CML/CLL	6/1
Non-malignant diseases	15 (23, 8%)
Donor type	
MSD	53 (84, 1%)
MUD	10 (15, 7%)
Stem-cell source	
Peripheral blood	55 (87, 3%)
Bone marrow	7 (11, 1%)
Cord blood	1 (1, 6%)
Conditiong regimen	
Myeloablative	58 (92%)
RIC	5 (8%)

ALL – acute lymphoblastic leukemia, AML – acute myeloblastic leukemia, NHL – non-Hodgkin lymphoma, CML – chronic myeloid leukemia, CLL – chronic lymphocytic leukemia, MSD – matched sibling donor, MUD – matched unrelated donor, RIC – reduced intensity conditiong

two different STR markers (D8S1179 and Penta E) and Amelogenin.

STR system selection. To obtain an informative STR system for chimerism analysis we performed a donor/recipient genotyping using a commercially available STR multiplex amplification kit PowerPlex 16 (Promega, Madison, WI, USA) that contains tetranucleotide STR markers D18S51, D21S11, TH01, D3S1358, FGA, TPOX, D8S1179, vWA, CSF1PO, D16S539, D7S820, D13S317, D5S818 as well as pentanucleotide STRs Penta E and Penta D and the primers specific for the Amelogenin locus [18]. The selection of informative STR loci was based on previously described experiences [15, 19], where in such loci donor and recipient alleles should be individually distinguishable (Figure 1.) and should not be in the range of stutter or echo peaks i.e. the donor and recipient derived alleles are 4 base pairs apart, as these peaks are PCR-generated artifacts [20, 21].

PCR amplification and fragment analysis. PCR amplification of commercial multiplex kit PowerPlex 16 system and selected monoplex kits PowerPlex16 monoplex D21S11, D3S1358, D8S1179, D16S539, D13S317, Penta E and Amelogenin (Promega, Madison, WI, USA) was carried out as recommended by the manufacturer, with a slight modification: using 10-50 ng of genomic DNA and BioThermStar DNA polymerase (Genekraft, Lüdinghausen, Germany) instead of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) in some reactions. The denaturation, annealing and extension cycles were programmed in Techgene thermal cycler (Techne Inc, Burlington, NJ, USA) as follows: preincubation 95°C for 10, 96°C for 1 minute, 10 cycles with 96°C/30 seconds (s), 60°C/30 s, 70°C/45 s and 14 and 18 cycles with 96°C/30 s, 60°C/30 s, 70°C/45 s for monoplex and multiplex kits, respectively and final elongation step performed at 60°C for 30 minutes [36]. For fragment analysis a mixture of 1 µl of the PCR product with 8,5 µl deionized formamide and 0,5 µl of the size standard ILS 600 (Promega, Madison, WI, USA) was prepared and was subjected to capillary electrophoresis in an ABI Prism 3100–Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The injection times varied between 4 and 36 seconds. Fragment length and fluorescence intensity were analyzed using GeneMapper Software (Applied Biosystems, Foster City, CA, USA) and the detection threshold was set for 50 RFU. Quantitative analysis using the fluorescence intensity given by the peak area were standardized using the general equation described by number of workers [10, 22]: % recipient DNA = (R1 + R2) x 100/ (D1 + D2 + R1 + R2), where R1, R2 are the recipient alleles peak area and D1, D2 are the donor alleles peak area under the curve. If an allele is shared, only the alleles that distinguish the donor from the recipient were used for calculation of chimerism [17]

Definition of chimerism status and outcome. The patients were divided on the basis of serial analysis by STR-PCR with approximately 1% sensitivity. Patients who showed no evidence of autologous – recipient DNA at any time post-transplantation follow-up were considered to have CC. Patients with both donor and recipient DNA in any of the samples analyzed were defined as having MC. Patients who showed an increase (5% or more) in the proportion of recipient DNA or who changed from CC to any level of MC between two consecutive assessments were referred as having increasing MC. Those patients with decreasing recipient DNA content (5% or more) or transforming from MC to CC in two successive samples were categorized as having decreasing MC [10, 23].

Statistical analysis. The probability of overall survival in two chimerism groups was estimated by the Kaplan-Meier method [24]. The observed differences in overall survival were assessed by the log-rank test. Bland and Altman graphical method was used as an indicator of agreement between expected and observed percentage of recipient DNA in artificial mixtures when three different systems were used in chimerism measurement [25]. To quantify the differences between the results of the three systems one sample t-test was used and the agreement between them was analyzed using the Friedman test with the post hoc Dunn's multiple comparison test. The level of significance was set to 0.05. Software used for the analysis was SPSS 13.0, GraphPad 4.0 and Microcal Origin 6.0.

Results

Chimerism status after SCT. In the whole group of 100 heterogeneous adult and pediatric patients we performed STR genotyping in 79 donor recipient pairs and found at least one informative STR marker out of 6 previously selected systems obtained in the commercially available multiplex kit in 78 cases (98, 7%). Neither system was informative in a monozygotic twins donor/recipient pair. Out of these 100 patients we further studied with a median follow up of 17 months (range 1 – 45 months) a cohort of 63 patients, whose blood samples were regularly analyzed for chimerism status, beginning one to two weeks after SCT. Those 37 patients that were excluded from the study did not have early post-transplant blood samples for chimerism analyses. In these sixty-three studied patients CC, maintained over the whole follow-up period, was detected in 24 (38, 1%), stable and decreasing MC in 28 (44, 4%) and increasing MC in 11 patients (17, 5%), all of whom were transplanted for a malignant disease. Patients with CC, stable and decreasing MC showed a significantly better (p 0,005) overall survival rate (0, 83), compared to those with increasing MC (0, 25) detected at any time after SCT (Figure 2). In the group of 11 patients with increasing MC eight experienced leukemia relapse. After detection of increasing MC they could not be offered immunotherapy to prevent relapse, because the time interval to overt hematological relaps was to short. Four of these 8 patients are still living, achieving a further remission, one of them successfully retransplanted. Three out of the 11 patients with increasing MC did not show any signs of leukemia

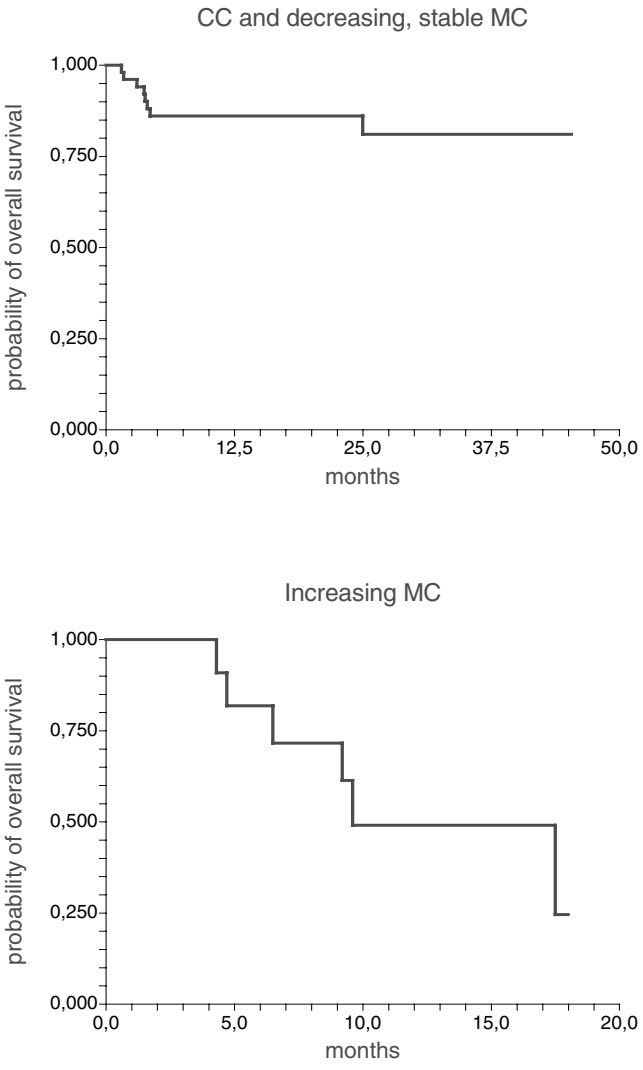

Figure 2. Kaplan-Meier analysis of overall survival in patients with different chimerism status after SCT

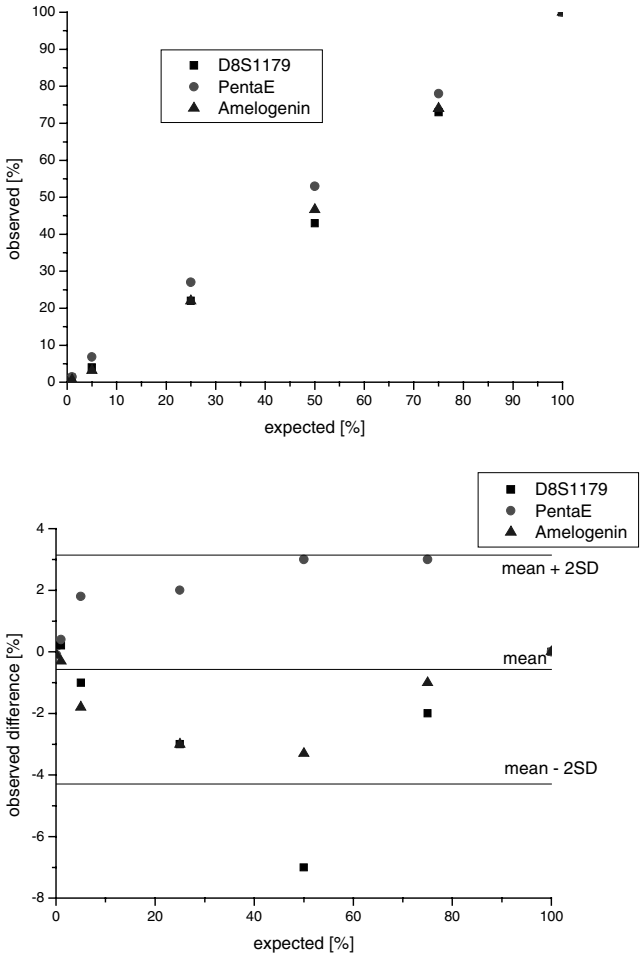

Figure 3. Comparison of the results obtained from chimerism quantification of three systems used (D8S1179, Penta E, Amelogenin) and the expected percentage in the artificial mixtures demonstrated by the Bland-Altman method. (A) Plot of the results obtained by D8S1179, Penta E, Amelogenin against the expected percentage. (B) Plot of the differences between the results against their mean.

recurrence, but two of them died one due to graft rejection and one as a consequence of chronic graft versus host disease (GVHD). One patient is still living after cessation of immunosuppressive therapy in complete remission revealing CC. Results summary is shown in Table 2. In all but 3 patients out of those ten who experienced relapse in the whole cohort it was diagnosed more then +100 days after SCT i. e. in the period when intervals of chimerism testing were already lengthened to 2-3 months. Two patients of the CC group experienced relapse; one showed CC a month before morphological relapse (no further sample was sent) and the other one revealed CC at the time of otherwise proved disease recurrence.

Sensitivity and quantification accuracy testing. Both STR markers (D8S1179 and Penta E) as well as Amelogenin used for sensitivity testing were able to detect recipient DNA in the DNA or blood mixtures of fictitious SCT containing 1% or more recipient DNA. We compared the accuracy of three different system used for chimerism detection in artificial mixtures according to Bland – Altman method that shows a graphical representation of the differences between expected (artificialy fabricated) and observed percentage of donor and recipient DNA in those three systems. The obtained results in artificial chimerism comprising 7 mixtures from 0,1% to 100% chimerism the observed and expected values were highly correlated and showed good agreement since most differences lay between the mean difference ± 2 SD (standard deviation) and were close to the expected percentage of donor and recipient DNA in the artificial samples (Figure 3). According to the

Table 2. Results summary

Characteristic	*Number (%)*
Donor recipient STR genotyping	79
Informative system	78/79 (98, 7%)
Patients in the studied cohort	63
Median follow up (month) (range)	17 (1-45)
Surviving patients	49/63 (77, 7%)
Chimerism status	
CC	24 (38, 1%)
stable and decreasing MC	28 (44, 4%)
increasing MC	11 (17, 5%)
CC, stable and decreasing MC group	52/63 (82,5)
Relapse	2/52 (3, 8%)
Relapse death	2/52 (3, 8%)
Non-relapse death	6/52 (11, 5%)
Increasing MC	11/63 (17, 5%)
Relapse	8/11 (72, 7%)
Relapse death	4/11 (36, 4%)
Non-relapse death	2/11 (18, 1%)
Surviving after relapse	4/11 (36, 4%)

Friedman test there were no statistically significant differences in the three systems used.

Discussion

Quantitative monitoring of chimerism after allogeneic SCT based on PCR amplification of microsatellite STR markers has become an important component of post-transplant surveillance of patients. It can document engraftment, predict graft failure or rejection, identify those patients who are at the highest risk to develop relapse and clarify the origin of the cells after relapse [4-6, 10, 25-27]. According to the changes in chimerism status after transplantation early implementation of immunotherapeutic measures such as rapid cessation of immunosuppression, donor lymphocyte infusion (DLI) with or without cytokine coadministration can be delivered as prophylaxis and seems to be highly efficacious in restoring CC and decreasing autologous cell contents [6, 11, 28]. Several methods have been developed to assess the level of chimerism and currently the most widely used is the PCR amplification of STR markers, which are short base pairs (bp) sequences distributed throughout the genome. Each STR marker is a system of many alleles, all sharing the basic structure of a repeat (2-8 bp in length), but differing in the number of tandem repeats of this sequence. They can be applied for follow up of virtually all patients and only small amounts of DNA are required for the test [20, 29, 30]. Recently, several investigators have used commercially available multiplex STR kits, formerly designed for forensic purposes, since they have a high degree of informativity and standardization [15]. The visualization and quantification of STR-PCR products is carried out with capillary electrophoresis using a DNA sequencer, overcoming rather cumbersome densitometry of gel bands when using gel electrophoresis [10, 16, 30-32].

Therefore, the present study evaluates our first results of chimerism testing with newly established method of PCR amplification of STR markers linked with quantitative assessment of amplified fragments with capillary electrophoresis. It seems that with the use of multiplex STR kit we could easily find at least one informative system: D21S11, D3S1358, D8S1179, D16S539, D13S317 or Penta E in 98, 7% of our patient population. This was observed also by other authors who evaluated informative markers for chimerism analysis, though for some, it was the most time-consuming step [15, 33-35]. We tried to select optimal markers to avoid the interference of confounding peaks as stutter peaks or echo peaks that would lead to false positive results, as described by others [20, 36-38]. The sensitivity of the individual markers to detect the minor component DNA was rather high, reliably detecting 1% or more of the minor DNA. As the quantification accuracy is concerned the artificial DNA mixtures measurements showed no statistical significant differences between the three systems (D8S1179, Penta E and Amelogenin) tested and they also showed good agreement between the expected (true) and observed values evaluated by other laboratories as well [16, 17, 30, 32]. Although increasing MC detected in serial sample analyses is described as a good predictive factor of relapse [5-7, 25, 39-41], we could not confirm its wholly predictive value. In our series, two relapses occurred without prior detection of recipient cells in peripheral blood and it appeared in 72,7% of patients (8/11) at a much too short interval before hematologic leukemia recurrence was proved, so no pre-emptive immunotherapy could be offered to avert relapse. This was observed also by other authors along with the fact that immunotherapy could not prevent leukemia relapse [23, 42-44]. In one of our patients with increasing MC withdrawal of immunosuppression led to prompt restoration of CC, what is in accordance with Bader et al [6]. Our results could in part be explained by small number of patients, in part by irregular blood sample collection irrespective of recommended timing for chimerism monitoring and partly it could reside on limited sensitivity of this technique. Therefore more sensitive approaches, such as real-time quantitative PCR of single nucleotide polymorphism might help to improve chimerism surveillance [45-47].

In conclusion, our study clearly shows that chimerism evaluation based on PCR amplification of polymorphic microsatellite STR markers is an readily applicable technique, informative almost for all patients, with good sensitivity and accuracy when used as recommended, providing a powerful tool in post-transplant decision making. Recently a large study on chimerism analyses involving 10 European countries, (Eurochimersm Consortium), has been completed and the results may help to increase standardization and optimization of this technique as well as appropriate timing of chimerism testing [9].

Despite the fact “Chimera” was described as an ugly monster [48], patients revealing different chimerism status after

SCT are the most stunning ones and are worth further studies to elucidate the potentials of serial chimerism monitoring.

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