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Monitoring of Chimerism in Rare Haematological Malignant Diseases after Allogeneic Haematopoietic Stem Cell Transplantation

Eva Hanusovska and Sabina Sufliarska

Abstract

Allogeneic haematopoietic stem cell transplantation (allo-hSCT) is one of the most important therapeutic options for patients with both malignant and non-malignant life-threatening rare disorders. Assessment of chimerism following allo-hSCT has been established as an indispensable tool for the clinical management of transplant recipients. The number of allo-hSCT among *CML* patients is decreasing due to tyrosine-kinase-inhibitor treatment. However, allo-hSCT in adult and paediatric patients with AML, ALL, and different non-malignant diseases is still increasing. For sex-independent patient chimerism monitoring, PCR-based short tandem repeat (PCR-STR) DNA markers with subsequent fragment analysis ('FA') and SYBR Green-based real-time PCR (SNPs or NPs markers of DNA) ('RQ PCR') were used. Specific features of chimerism assessment in non-malignant (n = 74) and malignant (n = 169) diseases were monitored by 'FA'. Complete and mixed chimerism was monitored also by SYBR Green-based real-time PCR method ('RQ PCR') (n = 188). By comparing the results of two chimerism monitoring methods ('FA' and 'RQ PCR') (n = 65), the higher sensitivity for the detection of the autologous DNA markers was observed by 'RQ PCR' (<1%) than 'FA' (1–5%). The lower detection limit of mixed chimerism could reveal an eventual relapse earlier. But the quantification of donor's DNA markers is more precise estimated by the FA.

Keywords: allo-hSCT, chimerism monitoring, diallelic indel polymorphisms, PCR-STR, rare diseases

1. Introduction

Bone marrow transplantation is widely used for many different kinds of haematological malignancies such as leukaemias/lymphomas and immunodeficiency which are rare diseases. Over the past decades, allogeneic haematopoietic stem cell transplantation (allo-hSCT) has gained increasing importance as a treatment

option for patients with both malignant and non-malignant life-threatening disorders in adult as well as paediatric patients. High risk leukaemias that are indicated for allo-hSCT are acute myeloid leukaemia (AML) [1], acute lymphoblastic leukaemia (ALL) and chronic myeloid leukaemia (CML) resistant to tyrosine-kinase inhibitor targeted treatment [2]. Also, myelodysplastic syndrome (MDS) and many other non-malignant disorders (bone marrow failure syndromes, haemoglobinopathies, immunodeficiencies and osteopetrosis) can profit from allo-hSCT [3].

Considerable progress has been made in the analysis of haematopoietic 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 centres performing allogeneic hSCT [4]. The term 'chimerism' was introduced in the field of medicine by Anderson et al. [5] to describe organisms whose cells derive from two or more zygote lineages. Close surveillance of chimerism within total peripheral blood leukocytes after an allogeneic hSCT seems an indispensable tool for the clinical management of transplant recipients [6]. In order to identify donor cells and even small amounts of residual host cells, many genetic methods have been established for this purpose. Cytogenetics and fluorescence in situ hybridization (FISH) analysis are the older ones and are applicable only in sex-mismatched transplantations where the proportion of X and Y chromosomes are detected [7]. The variability between individuals can be found both on the phenotype and on the genotype levels, especially in non-coding areas of the DNA. The later can be used not only in the population genetics, evolutionary studies and forensic and paternity proofs but also in the medicine as appropriate DNA informative markers to identify the donor and the recipient (host; patient) on the molecular level and monitor chimerism after allo-hSCT. For sex-independent patient chimerism monitoring, the PCR-based analyses of highly polymorphic short tandem repeats (STR; PCR-STR) DNA markers with subsequent fragment analysis ('FA') on Genetic Analyser are frequently used [8, 9]. Single-nucleotide polymorphism (SNP) or nucleotide polymorphisms (NPs) assessment by relative quantification SYBR Green-based real-time PCR, ('RQ PCR') Real-time PCR and semi-nested real-time PCR are used less [10–12], but due to increased interest in diallelic insertion/deletion polymorphisms 'DIPs' [13, 14], many new commercially kits for chimerism monitoring are available. In spite of the different analytical approaches to detect post-transplant chimerism, it seems to be useful to explain some common features. The term 'complete chimerism' (CC) expresses the status, where only the donor genotype is detected in the patient blood sample after allo-hSCT by the certain method. The term 'mixed chimerism' (MC) expresses the status, where both donor and recipient (host) genotypes are detected in the patient blood sample after allo-hSCT by the certain method. However, the post-transplant chimerism is a dynamic process. Several days, weeks, or months after allo-hSCT, the mixed chimerism is usually slowly changed to complete donor chimerism named 'decreasing mixed chimerism'. Vice versa in the case of a relapse when autologous haematopoiesis is appeared often the complete chimerism becomes to the status of mixed chimerism named 'increasing chimerism'. The coexistence of donor and recipient haematopoiesis for months or a longer period (especially in non-malignant disorders) is named 'stable mixed chimerism' [3].

In following chapters, we would like to describe not only two different DNA molecular methods ('FA' and 'RQ PCR'), which have been used in chimerism monitoring after allo-hSCT in our laboratories, but also its comparison from our results.

2. PCR-STR DNA markers in chimerism monitoring by 'FA'

2.1 Introduction

Quantitative monitoring of chimerism after allo-hSCT based on PCR amplification of microsatellite STR markers has become an important component of post-transplant surveillance of patients. Each STR marker is a system of many alleles, all sharing the basic structure of a repeat (2–8 bp in length), (4–5 bp in our study) 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 to estimate to donor/recipient chimerism after allo-hSCT [15–17]. Quantitative chimerism monitoring 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 [18–23].

2.2 Methods

The PCR-based STR (PCR-STR) DNA markers were amplified using fluorescently labelled allele-specific primers, and different amplicons were separated by capillary electrophoresis on Genetic analyser. The direct quantification of donor and recipient PCR-STR DNA markers was provided by fragment analysis (FA) of GeneMapper software (Applied Biosystems) [20, 24–28].

2.3 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 months thereafter during the first year according to the transplantation centre. During the second year, the frequency was reduced to twice a year, only if the clinical situation warranted, more frequent chimerism analyses were performed.

2.4 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. 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, e.g. D18S51, D21S11, TH01 and D3S1358, as well as pentanucleotide STRs Penta E and Penta D and the primers specific for the Amelogenin locus [27, 28].

2.5 PCR amplification

About 10–50 ng of genomic DNA and BioThermStar DNA polymerase (GeneCraft, 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 min, 96°C for 1 min, 10 cycles with 96°C/30 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 min.

2.6 Fragment analysis

For fragment analysis, a mixture of 1 μl of the PCR product with 8.5 μl deionised 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 Analyser (Applied Biosystems, Foster City, CA, USA). The injection times varied between 4 and 36 s. Fragment length and fluorescence intensity were analysed using GeneMapper Software (Applied Biosystems, Foster City, CA, USA), and the detection threshold was set for 50 RFU. The selection of informative STR loci was based on previously described experiences [29, 30], where in such loci donor and recipient alleles should be individually distinguishable [15, 18].

2.7 Chimerism in adult malignant disease following hSCT monitored by 'FA'

2.7.1 Cohort

The cohort consisted of 169 adult and paediatric malignant patients from three Slovak bone marrow transplantation centres who underwent allo-hSCT between the years 1993 and 2010, and chimerism analysis of polymorphic STR markers was performed by 'FA'. A prospective evaluation of chimerism was performed in 171 patients transplanted after the year 2003 also by 'FA' from fresh or frozen DNA samples which were available.

2.7.2 Methods

The method included PCR-based analysis (with fluorescent allele specific primers) of short tandem repeats (PCR-STR) markers by fragment analysis ('FA') following capillary electrophoresis in Genetic analyser.

2.7.3 Results

The chimerism analysis of transplant patients was done for the first time no later than 1 month after allo-hSCT and further on 1–4 weekly intervals up to day +200 after transplantation and then according to the chimerism result and the timing of clinical controls. Patients who showed no evidence of autologous (recipient) DNA at any time in the post-transplantation follow-up were considered to have complete chimerism (CC). Patients with both donor and recipient DNA in any of the samples analysed were defined as having mixed chimerism (MC). An example of patient's mixed chimerism is presented in **Figure 1**. The average quantity of donor alleles 30.2 and 31.2 is 77%. The average quantity of recipient's alleles 16 and 25.2 is 23%, which means patient's mixed chimerism is 23% (MC = 23%). 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 categorised
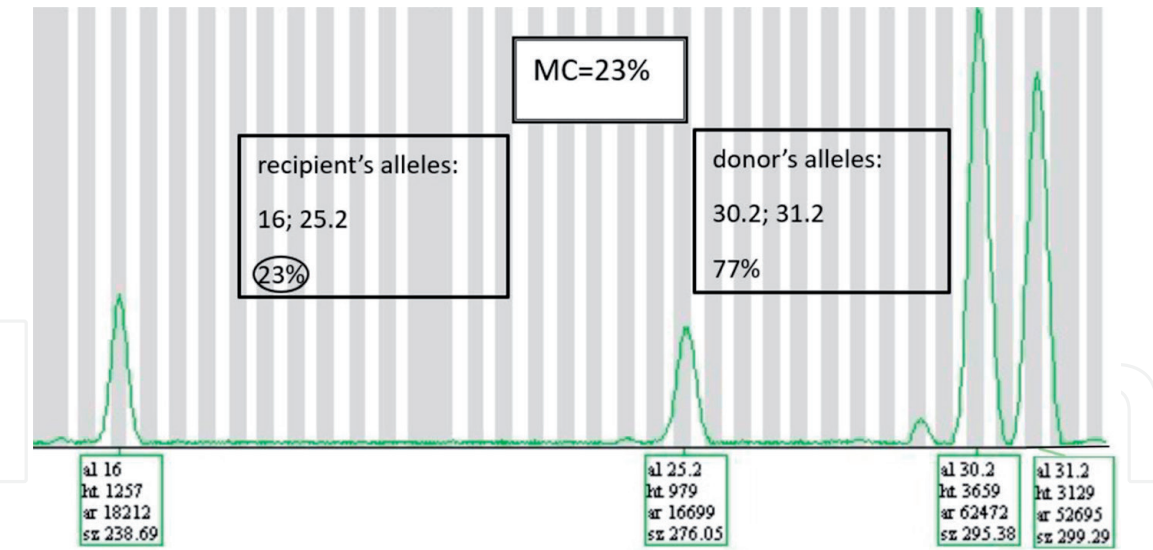
Figure 1.
Mixed chimerism (MC) in a patient after allo-hSCT

as having decreasing MC [26, 31]. Patients from our cohort with CC, stable MC, and decreasing MC showed a significantly better (p = 0.005) overall survival rate (OSR = 0.83) after allo-hSCT (**Figure 2**), compared to those with increasing MC (OSR = 0.25) after allo-hSCT (**Figure 3**) detected at any time after allo-hSCT.

2.7.4 Conclusion

Our observation shows that chimerism analysis gives clear information about engraftment, its failure, or graft rejection but is not a sufficiently sensitive method to detect an imminent relapse in acute lymphoblastic leukaemia patients. According to the changes in chimerism status after transplantation, early implementation of immunotherapeutic measures such as rapid cessation of immunosuppression and 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 [18, 27, 32].
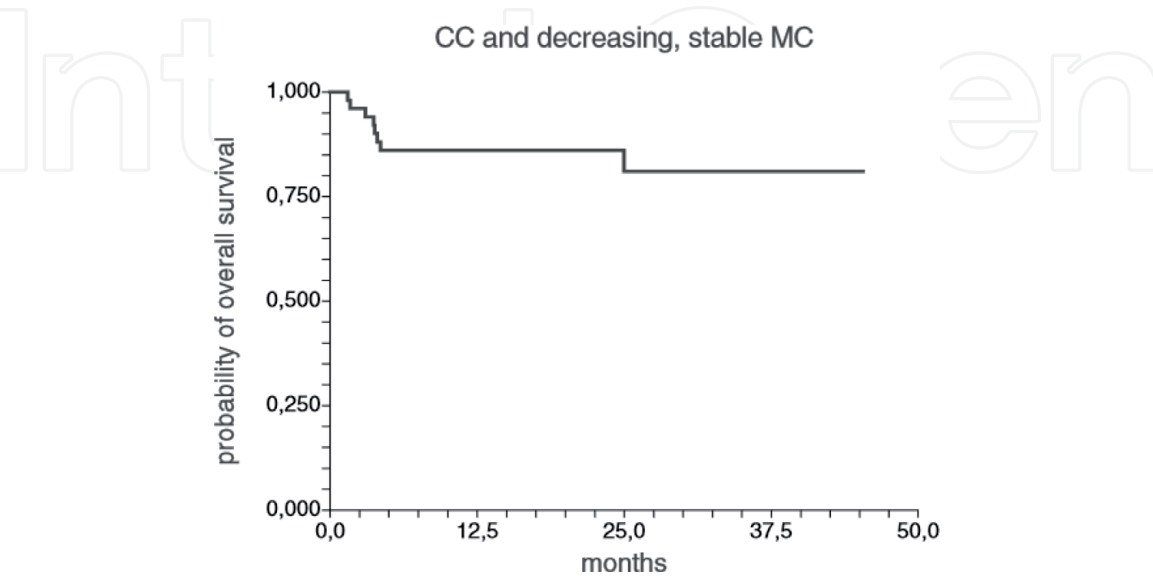

Figure 2.
Kaplan-Meier analysis of overall survival in patients with complete chimerism (CC), decreasing, stable mixed chimerism (MC) after allo-hSCT.
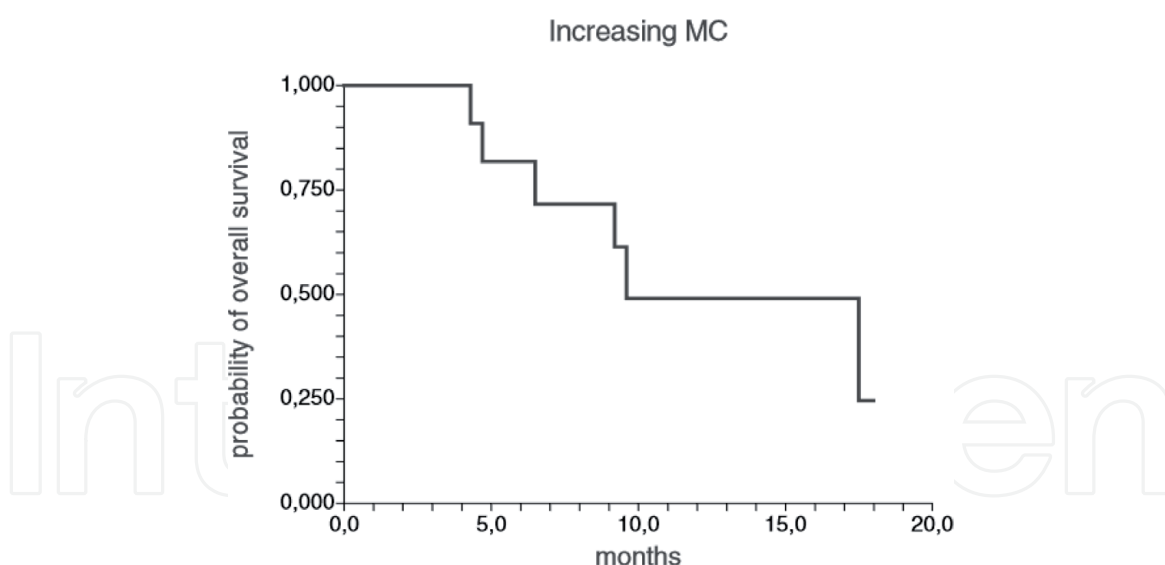
5

Figure 3.
Kaplan-Meier analysis of overall survival in patients with increasing mixed chimerism (MC) after allo-hSCT

2.8 Specific features of chimerism assessment in non-malignant diseases monitored by 'FA'

2.8.1 Introduction

Allo-hSCT is a well-established treatment not only for high risk leukaemias and myelodysplastic syndrome (MDS) but also for several non-malignant diseases, including acquired and inherited bone marrow failure (BMF) syndromes like aplastic anaemia (AA) and Fanconi anaemia (FA), immunodeficiencies like severe combined immunodeficiency (SCID), Wiskott–Aldrich syndrome (WAS), chronic granulomatous disease (CGD), haemoglobinopathies and metabolic disorders, mostly transplanted in childhood. The aim of transplantation in these diseases is to achieve sustained engraftment of donor stem cells to improve haematopoietic function, provide immune competence and increase or normalise enzyme deficiency. It can completely transform the lives of children for whom life expectancy or quality of life would otherwise be very poor. Although complete donor haematopoiesis is a desirable outcome of SCT in malignant disorders, complete replacement of recipient's haematopoietic system is not considered necessary to improve the underlying disease state in patients with non-malignant disorders [33, 34].

2.8.2 Cohort

The cohort consisted of 74 different adult and paediatric non-malignant patients from three Slovak bone marrow transplantation centres who underwent allo-hSCT.

2.8.3 Methods

The method included PCR-based analysis (with fluorescent allele specific primers) of short tandem repeats (PCR-STR) markers by fragment analysis ('FA') following capillary electrophoresis in Genetic analyser. Surveillance of chimerism was done within total peripheral blood leukocytes or as lineage-specific chimerism in selected T cells, B cells and myeloid cells after an allogeneic SCT according to Park et al. and Thiede [35, 36].

2.8.4 Results

Definition of chimerism, as it is described above considers a patient to have CC, when he does not show any evidence or less than 1% of autologous—recipient DNA at any time after allo-hSCT. Patients with both donor and recipient DNA (that increased 5% or more) in any of the samples analysed were defined as having MC. Split chimerism is present if one or more leukocyte lineages are of host and one or more leukocyte lineages are of donor origin [4, 36, 37].

As other studies mentioned above, also our results show there is a relationship between chimerism status and clinical course as well as outcome of allogeneic SCT in non-malignant diseases. From our cohort, 75% (55 patients) reached complete chimerism (CC) monitored by 'FA' and 25% (19 patients) mixed chimerism on different levels. Most of the patients with CC and decreasing or stable MC are alive doing well and are in remission, with a median follow-up time of 4.3 years. All of the patients with high MC (>40% or increasing MC) experienced transplant rejections and almost half of them died. Patients with CC had a higher risk of acute graft-versus host disease GVHD compared with MC patients.

2.8.5 Conclusion

Therefore, mixed chimerism (if there are still low levels of recipient cells) may be welcomed in these patients, as it reflects a decreased allo-response with less acute GVHD. Reduced intensity conditioning (RIC) was more often associated with decreasing or low stable MC compared to myeloablative conditioning, but importantly high MC was not different when using reduced intensity conditioning than with myeloablative conditioning [33, 34].

3. SNP and NP DNA markers in chimerism monitoring by 'RQ PCR'

3.1 Introduction

Chimerism monitoring allows the characterisation of the haematopoietic stem cell origin in the recipient's blood or bone marrow after allo-hSCT. The DNA identification of the person is mainly based on the DNA polymorphisms, which include single-nucleotide polymorphisms (SNPs) due to nucleotide substitutions and insertion or deletion of one or more nucleotides (indels). The late can be multiallelic (STR) and diallelic (biallelic). The extensive bibliographical search for human diallelic indels and its basic properties were determined and selected for chimerism monitoring [13, 38]. The sensitive quantitative real-time PCR analysis using indel polymorphisms can be the useful tool to predict relapse in leukaemia patients after allo-hSCT [39].

3.2 Complete and mixed chimerism monitoring by SYBR green-based real-time PCR method ('RQ PCR')

Tens of biallelic (diallelic) nucleotide polymorphisms specific for the donor and the recipient can be detected by the method of allele-specific real-time PCR.

3.2.1 Cohort

The cohort consisted of 188 patients from University Hospital Bratislava, Slovak Republic and 188 donors (111 from relatives and 77 from national and world registers of the bone marrow).

3.2.2 Methods

The DNA was isolated (NucleoSpin Blood, Macherey-Nagel) from peripheral blood leukocytes (centrifugation 3000 × g/10 min) before allo-hSCT from both donor and recipient and 30, 100, 180 and 365 days after allo-hSCT only from recipient (patient). Concentration of the DNA was measured on NanoPhotometer, Implen and samples were diluted to 10 ng/μl. To get allogenic and autologic informative markers for quantification after allo-hSCT, screening of donor and recipient DNA samples before allo-hSCT was essential. Three 1 μM allele-specific primers (two forward and one reverse or one forward and two reverse) for 11 biallelic nucleotide polymorphic markers were localised on 1st, 5th, 6th, 9th, 11th, 17th, 18th, 20th and X chromosome. Also, two pairs of specific primers (forward and reverse) for monoallelic DNA marker on Y chromosome (amelogenin) and for endogenic control gene GAPDH were used (**Figure 4**) [40]. The amounts for one-well allele-specific PCR reaction on 96-well plate were: 12.5 μl Power SYBR Green (PCR Master mix, Power SYBR green; Applied Biosystems), 1.25 μl 1 μM forward-primer (A or B) and1.25 μl 1 μM reverse primer (C) or vice versa (Sigma Genosys), 5 μl DNA (10 ng/ul), 5 μl DNase, RNase free water to final volume 25 μl. Thermal profile of 7300 Real Time PCR System (Applied Biosystems) for allele-specific PCR: 50°C/2 min, 1×; 95°C/10 min, 1×; (95°C/0.15 min, 60°C/1 min)/50×; and dissociation of amplicons with incorporated SYBR green: 94°C/0.15 min; 60°C/0.30 min; increasing 1°C/min; 95°C/0.15 min. Amplification curves from real-time PCR with following dissociation curves revealed type A, or B of marker (M) allele (MA or MB). For each donor/recipient pair, there are different informative markers for relative quantification and chimerism estimation after allo-hSCT. Relative quantification was calculated and evaluated from amplification plots of the individual PCR reactions on 96-well plate by the gene expression Study software (7300 System SDS Software, Applied Biosystems). Amplification plot of each informative marker before alloTKB was used as the calibrator for relative quantification of measured marker and amplification plot of gene GADPH was used as endogenous control [40].

3.2.3 Results

In most of screened donor/recipient pairs, we have found 2–3 informative autologous and allogeneic markers. An example of donor and recipient (patient) screening for informative NPs DNA markers (M1–M12) by SYBR green-based real-time PCR amplification and following dissociation curves is presented in **Figure 5**. For this donor/recipient pair, recipient's marker 3B (M3B) can be considered as informative autologous marker and can be used for chimerism monitoring after allo-hSCT. Only in three cases, we did not find any of the informative autological DNA markers, which are most convenient (detection <1%) for the measurement of micro chimerism. After allo-hSCT, informative DNA markers found in samples of patient's peripheral blood of patients were quantified. The relative amounts (expressed in %) were evaluated from threshold cycle (cT) values of amplification curves and analysed by Study Software, previously used for a relative quantification of the gene expression. The results of the DNA marker quantification measurements were obtained from (relative quantification) RQ and simultaneously from the charts, which had been constructed automatically [41] and transformed (**Figure 6**). In this figure, chimerism monitoring of AML patient after allo-hSCT is expressed. All patients were regularly monitored, and from the quantification of autologous informative DNA markers, mixed (MC) or

Figure 4.
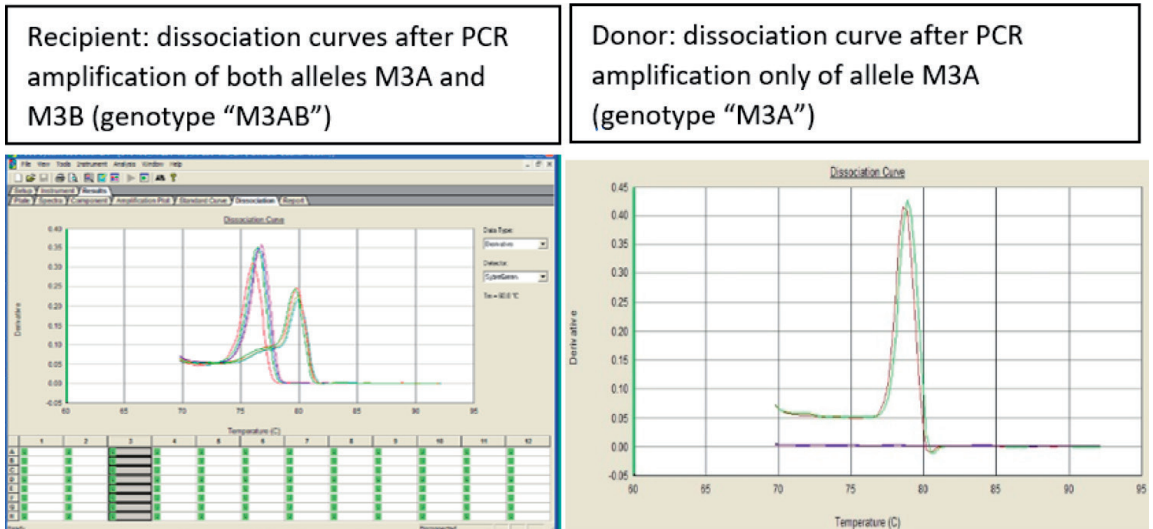*Localisation of DNA markers (M1-M12) (11NPs).*

Figure 5.
An example of screening DNA markers M1-M12 by SYBR green based real-time amplification and following dissociation curves. Recipient's marker 3B can be used for chimerism monitoring after allo-hSCT.

complete (CC) chimerism was evaluated. Identification of donor allogenous informative markers have evaluated only semiquantitatively. Cutoff for CC was 0.01% with relative quantification sensitivity RQ = 0.0001. Special time-dependent charts for each patient were archived and sent to doctors in appropriate transplantation units.

3.2.4 Conclusion

We have implemented the method of DNA diagnostics for the detection of micro chimerism (add to cytogenetics and FISH previously used) after allo-hSCT, which is independent from the sex of donor and recipient and reaches sensitivity of <1% (0.01%). The method of relative quantification by real-time PCR is currently routinely used in our clinical practice and can serve clinician as another factor for the patients monitoring after haematopoietic stem cell transplantation and his early intervention if necessary.
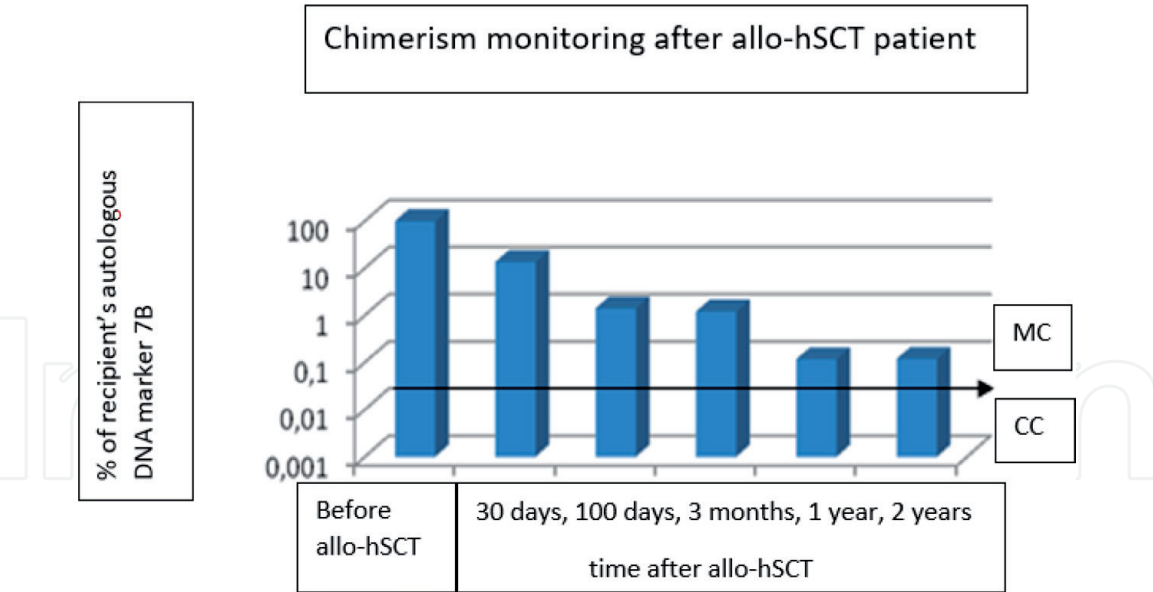
Figure 6.
Chimerism monitoring of AML patient after allo-hSCT by SYBR green based real-time PCR relative quantification method. MC, mixed chimerism; CC, complete chimerism.

3.3 Different malignant and non-malignant diagnosis in our study and their donors provided by 'RQ PCR'

3.3.1 Results

In our diagnostic laboratory, we have analysed samples from patients before and after allo-hSCT with different malignant and non-malignant diagnoses (**Figure 7**).

During the last decades, the number of allo-hSCT among *CML* patients is decreasing due to the drug-targeted treatment and transplantation is mostly indicated in the case of resistance to targeted tyrosine-kinase-inhibitor treatment. In our study, only 9 CML patients were transplanted from the cohort of 188 different malignant and non-malignant diagnosis (patients from University Hospital Bratislava, Slovak Republic), which is only 5%. The majority of allo-hSCT were provided and chimerism was monitored in AML patients (45%); then, ALL patients were 31% and MDS with others non-malignant diagnoses were 19% (11 + 8).

The selection of a donor is a critical element contributing to the success of haematopoietic cell transplantation. Possible donors for allo-hSCT can be HLA identical, haploidentical or mismatched (sibling, relative or unrelated donor). In our study, there were 188 donors (111 from relatives and 77 from Slovak National Bone Marrow Donor Registry and world registers of the bone marrow donors) and 188 recipients (**Table 1**). About 95% haemopoietic stem cells were obtained from peripheral blood by its drug mobilisation. Only 5% stem cells were from the bone marrow.

3.3.2 Conclusion

From our results, it is obvious that assessment of sex-independent donor/recipient method based on SYBR green real-time relative quantification RQ PCR) for chimerism monitoring has been very important due to cohort of different gender donor/recipient pairs. This molecular method has completed cytogenetics and FISH methods used previously for chimerism monitoring. The advantage of 'RQ PCR' compared to methods mentioned above is not only in its higher diagnostic and analytical sensitivity and sex-independent monitoring but also in the fact
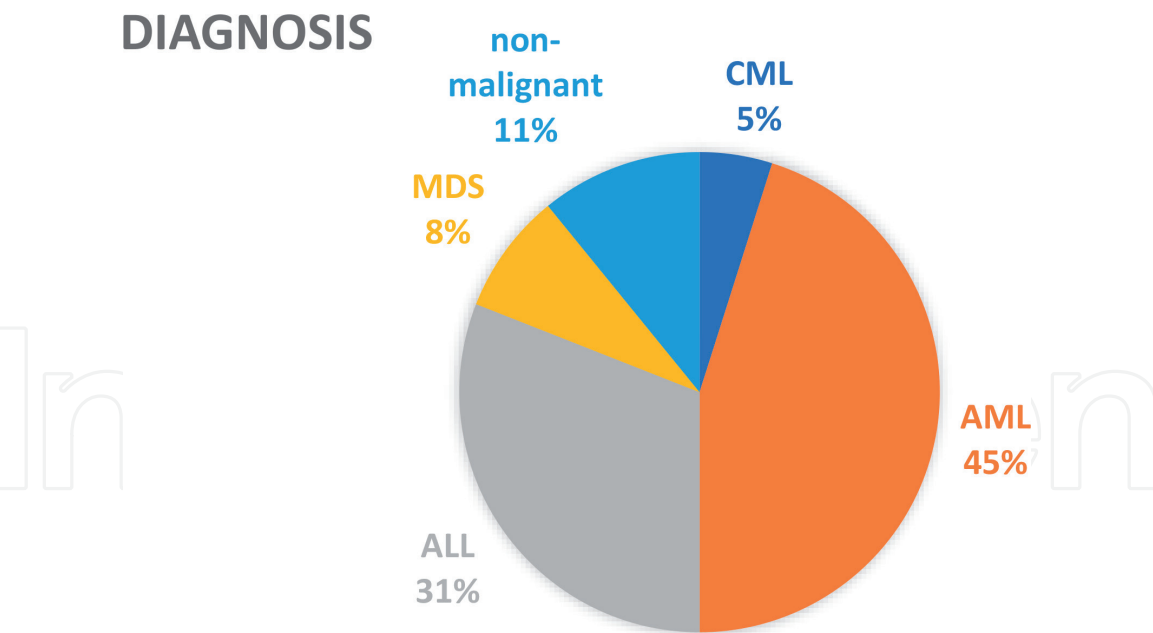
Figure 7.
Different malignant and non-malignant diagnosis in process of allo-hSCT

Types of recipient/donor in allo-hSCT	
Total number of transplant patients-recipients	188
Total number of donors	188
Related donors for allo-hSCT	**111**
Female/female	15
Male/male	42
Sex-mixed	54
Unrelated donors (MUD) for allo-hSCT	77
Female/female	9
Male/male	33
Sex-mixed	35

Table 1.
Types of recipient(patient)/donor variants in the process of allo-hSCT.

that almost 50% of AML patients has not genetic mutation marker to distinguish increasing mix chimerism and no indicator for relapse of primary patient disorder before allo-hSCT.

4. Chimerism evaluation by two different DNA marker sets and molecular methods 'FA' and 'RQ PCR'

4.1 Introduction

The aim of our study was to compare patient's chimerism monitoring by two different DNA marker type sets and molecular methods 'FA' and 'RQ PCR' measured in parallel after allo-hSCT [40, 42]. The subject matter of the first PCR-STR method 'FA' is explained in chapter '2—PCR-STR DNA markers in chimerism monitoring by "FA"'. Informative STR DNA markers were amplified with special fluorescent

labelled primers by PCR. Amplicons were separated by the capillary electrophoresis on genetic analyser and then evaluated by fragment analyses GeneMapper software. The basis of the second method 'RQ PCR' (SYBR green-based relative quantification by real-time PCR) to be compared is discussed in the chapter "3—SNP and NPs DNA markers in chimerism monitoring by RQ PCR". Informative SNP and biallelic NP (including DIPs—IN/DEL polymorphic markers) DNA markers were obtained from donor's and recipient's DNA before transplantation by SYBR green-based real-time PCR amplification following dissociation curves analysing screening. Post allo-hSCT chimerism monitoring is provided by relative quantification (RQ-PCR) of SYBR green-based real-time PCR of SNP or NPs informative DNA markers and evaluated by gene expression software. The relative quantity of the donor's and recipient's DNA markers is proportional to total leukocytes and can be expressed as mixed (MC) or complete chimerism (CC) in post-transplant patients.

4.2 Cohort

The cohort consisted of 65 AML, ALL and CML patients from Department of Haematology and Transfusion, Comenius University Medical School Bratislava and 65 donors from national and international bone marrow registers according their HLA compatibility to the individual patient.

4.3 Methods

Whole peripheral blood samples were collected for DNA extraction from both the donor and recipient before transplantation in order to determine an informative marker for two molecular methods mentioned previously. The blood samples of leukaemia patients (AML, ALL and CML) after allo-hSCT (N = 65 pairs) were collected at regular time periods (before allo-hSCT and 30 days, 100 days, 6-month, 1 year, 2 years and 3 years after allo-hSCT) at the Department of Haematology and Transfusion, Comenius University Medical School Bratislava. Isolation of the DNA and chimerism testing was provided in two diagnostic laboratories: Laboratory Diagnostics Medirex, Bratislava and Bone Marrow Transplantation Unit, Bratislava, Slovak republic, EU. RQ-PCR was performed by the real-time PCR system using SYBR green and 12 pairs of specific primers for two allelic variants of DNA polymorphism and GAPDH as endogenous gene control. The cT (threshold cycle) values of informative patient's DNA markers before allo-hSCT were used as calibrator for the relative quantification. The PCR reaction mixtures and DNA were placed on 96-well reaction plates in duplicates separately, not as multiplex amplification set. Relative quantification was evaluated by gene expression software study [40]. The PCR-STR analysis was performed using commercially available STR multiplex amplification kits with fluorescently labelled PCR primers. The quantification of donor and recipient DNA marker's signal was provided by comparing the fluorescence intensity given by the peak area of analysed fragments [20].

4.4 Results

We screened 65 related and unrelated donor/recipient pairs by both molecular methods, and we found at least one informative marker for each laboratory approach mentioned above. The parallel quantifications of DNA (two different informative DNA marker sets used) were provided by both methods, and the estimated chimerism was compared at the same time period after the allogeneic haematopoietic stem cell transplantation. We found that our results were identical only in 2% and the discrepancy was noticed also in 2% between the two methods

used. In the case of 1–50% mixed chimerism (MC), similar results were obtained. However, complete chimerism (CC) estimated by the fragment analysis was evaluated as mixed chimerism (MC) by the real-time PCR in 94% patients, mainly in the first half of a year of the post-transplantation monitoring. The example of the parallel monitoring of one patient is shown in **Figure 8**. From this presented results of patient chimerism monitoring, we can see that 30 days after allo-hSCT mixed chimerism was detected by both methods used (FA-MC and RQ PCR-MC). Also 1, 2 and 3 years after allo-hSCT, both methods showed identical complete chimerism results (FA-CC and RQ PCR-CC). But the discrepancy was revealed 100 days after allo-hSCT where complete chimerism was detected by the method FA (FA-CC), but simultaneously mixed chimerism was detected by RQ PCR method (RQ PCR-MC) in the same patient sample.

4.5 Conclusion

The parallel chimerism monitoring of post allo-hSCT leukaemia patients was provided for 3 years. Discrepancy between complete chimerism (CC) detected by the fragment analysis (FA) of PCR-STR DNA markers and mixed chimerism (MC) detected by the real-time PCR of SNP and NP DNA markers was due to the different sensitivity of two methods used. It is also important to note that in different molecular diagnostic approaches also, two different DNA marker sets were used. RQ PCR had the higher sensitivity (<1%) for the detection of the autologous DNA markers than FA (1–5%), so it is better for earlier revealing of eventual relapse. On the other hand, the quantification of donor's DNA markers is more precise estimated by the FA. Both methods compared above are suitable for chimerism assessment after
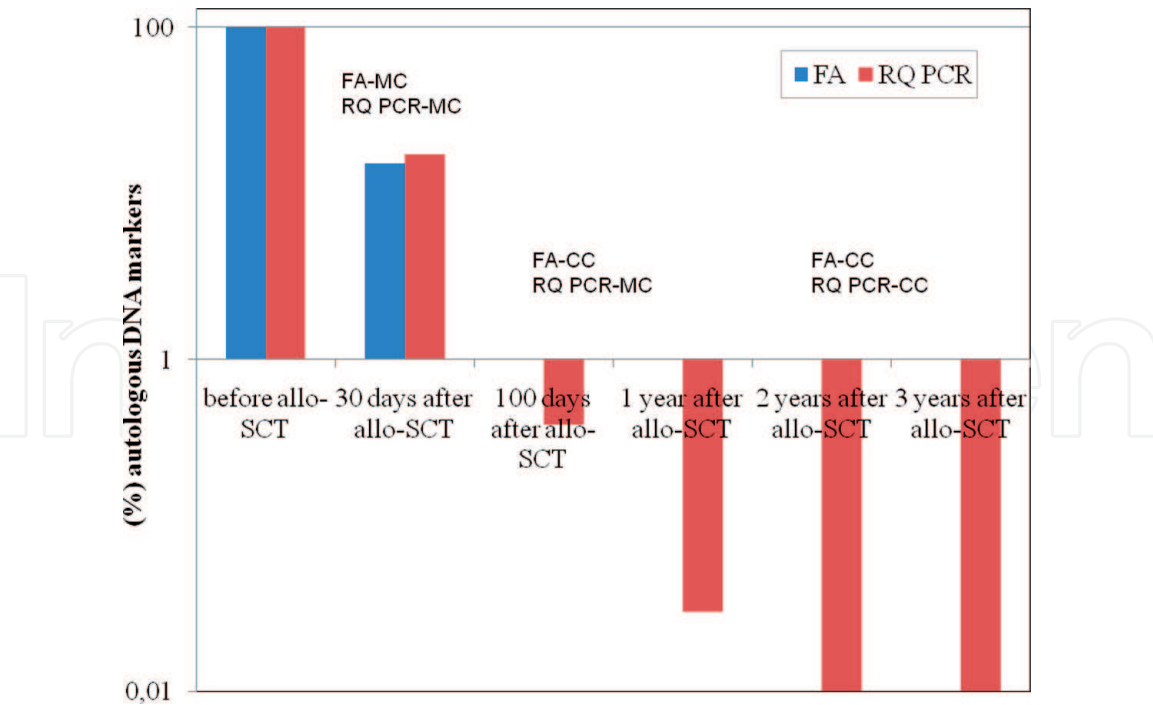

Figure 8.
Parallel monitoring of chimerism of one leukaemia patient after haematopoietic allogeneic stem cell transplantation (allo-hSCT) by two DNA molecular methods in different time periods. FA, analysis of PCR-STR DNA markers by fragment analysis following capillary electrophoresis on Genetic analyser; RQ-PCR, analysis of SNP and NPs DNA markers by relative quantification of SYBR green-based Real-time PCR; CC, complete chimerism (only donor's DNA markers detected in patient); MC, mixed chimerism (recipient's -autologous and donor's DNA markers detected in patient). 100 days after allo-SCT complete chimerism was detected by the method FA (FA-CC), but simultaneously mixed chimerism was detected by RQ PCR method (RQ PCR-MC) due to its different sensitivity.

the allogeneic haematopoietic stem cell transplantation, although nowadays many new kits with different DNA marker sets (STR, SNP, NPs and DIPs) for chimerism monitoring are available.

5. Conclusions

A number of studies have shown that chimerism evaluation based on PCR amplification of polymorphic microsatellite STR markers is a readily applicable technique, informative almost for all patients, but less sensitive then real-time PCR of SNP and NPs DNA method. It is important to notice that complete, mixed chimerism, decreasing chimerism, and increasing chimerism are only the relative terms, because different laboratories have their own criteria to differentiate between complete donor chimerism and mixed decreasing chimerism, based on the method that is used, its sensitivity and local policies [43–45]. However, both provide a powerful tool in post-transplant decision making. They 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. According to the changes in chimerism status after transplantation, early implementation of immunotherapeutic measures such as rapid cessation of immunosuppression and 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.

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Conflict of interest

The authors declare no conflict of interest.

Abbreviations

ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
allo-hSCT	allogeneic haematopoietic stem cell transplantation
AA	aplastic anaemia
BFM	bone marrow failure
CC	complete chimerism
CGD	chronic granulomatous disease
CML	chronic myeloid leukaemia
cT	threshold cycle
DIPs	diallelic insertion/deletion polymorphisms
DLI	donor lymphocyte infusion
DNA	deoxyribonucleic acid

FA	fragment analysis
GVHD	graft versus host disease
MC	mixed chimerism
MDS	myelodysplastic syndrome
NPs	nucleotide polymorphisms
OSR	overall survival rate
PCR	polymerase chain reaction
RQ-PCR	relative quantification real-time PCR
SCID	severe combined immunodeficiency
SNPs	single-nucleotide polymorphisms
STR	short tandem repeats
SYBR	Green intercalation dye
WAS	Wiskott–Aldrich syndrome

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