Review

Acta Hæmatologica

Strategies and Clinical Implications of Chimerism Diagnostics after Allogeneic Hematopoietic Stem Cell Transplantation

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Abstract

Analysis of donor chimerism has become a routine method for the documentation of engraftment after allogeneic hematopoietic stem cell transplantation (HSCT). In recent years several groups have also focused on the application of this technique for the detection of relapsing disease after allogeneic HSCT. This review addresses technical issues (sensitivity, specificity) and discusses the advantages and limitations of methods currently used for chimerism analysis and their usefulness for the detection of MRD. In addition, the potential impact of novel procedures, e.g. subset chimerism or real-time PCR-based procedures, is discussed.

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Introduction

After allogeneic blood stem cell transplantation, a coexistence of the host and donor lympho- and hematopoietic system will develop. This period, which is temporary in the majority of successful stem cell transplants, is referred to as mixed chimerism, whereas complete chimerism denotes the situation, when all cell lineages are reconstituted by the donor. The evaluation of chimerism after stem cell transplantation has been of central interest since this treatment has been used clinically. Several techniques have been applied for this purpose during the last 30 years, including cytogenetics, isoenzyme analysis and blood group phenotyping [for a review, see 1]. A major improvement in the clinical applicability was made when differentiation of sex chromosomes using fluorescence in situ hybridization (FISH) became possible [2, 3]. This method allows rapid and quantitative evaluation of engraftment; however, it is obviously restricted to the approximately 50% of patients transplanted from a sex-mismatched donor. The invention of the polymerase chain reaction (PCR) was a key step which laid the fundament for the development of modern molecular diagnostics [4]. In the field of chimerism diagnostics, several PCR-based procedures have been developed for the evaluation of engraftment [5–16]. Most of these assays rely on the amplification of highly polymorphic repetitive DNA sequences, i.e. short tandem repeats (STR) or a variable number of tandem repeat (VNTR) sequences.

Besides the documentation of engraftment, detection of reappearing leukemic cells has become a key issue in chimerism diagnostics. Numerous publications deal with this application. This review focuses on the use of chimerism analysis for the detection of minimal residual leuke-

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Table 1. Comparison of different methods for chimerism analysis

Method	Sensitivity[1], %	Advantages	Disadvantages	Ref.
XY-FISH	0.1	Quantitative relatively sensitive, fast, standardized technique	Restricted to sex mismatch	2, 3
Real-time PCR	0.1–0.0001	Quantitative, highly sensitive	False positive results possible in SNP-based procedures	23–25
Red cell phenotyping		Fast sensitive	Long latency makes it unsuitable for rapidly proliferating diseases	26
STR or VNTR-PCR	5–1	Informative, low amounts of material, rapid	Moderate sensitivity	5–16
YX-FISH and immuno-histochemistry (FICTION)	0.1–0.001	Sensitive, cell line specific	Labour intensive, restricted to sex mismatch	27–30
STR-PCR + cell sorting	0.1–0.0001	Sensitive, cell line specific	Labor intensive, requires specific equipment	31–34

* The lower level of host cells detectable is denoted.

mia and addresses several questions which may be important in this context: (1) Are the current methodologies for chimerism analysis appropriate for the detection of minimal residual disease (MRD)? (2) If so how can they potentially be further improved? (3) If not, which alternatives can be found to sufficiently solve the problem?

Methods for Chimerism Analysis and Their Sensitivity

The first of these questions is especially important. The term MRD describes a state of leukemic burden which is below the threshold detection level of conventional measures, i.e. morphology. This standard method for assessment of response towards chemotherapy has, by definition, a limit of detection for leukemic blasts in the bone marrow of 5% [17]. Thus any procedure which can measure cells below this level may be suitable to detect leukemic cells. But what level of sensitivity should we approach? Generally one might wish to know precisely which level of MRD has been reached. Thus, it is much more important to quantitatively assess the level of residual leukemia than to deal with merely qualitative analyses. The level of detection achievable can be illustrated by comparing several techniques which are currently used for the detection of residual leukemia after treatment. For example, FISH detection of chromosomal translocations has a level of sensitivity between 0.1 and 8% depending

on the technique used and the specific translocation investigated [recent review in 18]. Flow-cytometric assays, monitoring aberrant antigen expression, might achieve considerably higher levels of sensitivity, between 0.01 and 1% [recent examples in 19, 20]. PCR, however, represents the most sensitive technique reported so far. The exponential amplification of specific target sequences facilitates a limit of detection between 0.1 and 0.01% cells. This sensitivity can be further increased if two consecutive rounds of PCR are applied, which routinely detects $1/10^5$ to $1/10^6$ cells, and which can be further increased and has been used even to demonstrate the presence of leukemia-associated translocations in healthy individuals [21]. The diagnostic message can be substantially augmented when quantitative PCR methods (real-time PCR) are applied, which gives information on the number of starting molecules [22]. Thus, taken together there is a broad range of methods for the detection of residual leukemia. The methods currently in use for chimerism analysis must be interpreted in this context, if discussing their value in the detection of MRD. Table 1 summarizes different techniques for chimerism analysis, their advantages and disadvantages as well as the reported levels of sensitivity.

Among these assays, STR-based methods are the most frequently used. These methods usually have a level of sensitivity between 1 and 5%, a range which allows the accurate and reliable monitoring of engraftment. In addition, there is evidence that at least in slowly proliferating

diseases like CML, this level may be sufficient for the routine follow-up. Several reports have shown that increasing mixed chimerism is a sensitive predictor of relapse in cases with persistent positivity for the bcr-abl fusion transcript [35, 36]. However, these data mainly originate from the era before real-time PCR for the bcr-abl mRNA has become widely available [37]. Today, the quantitative information on the bcr-abl copy numbers is regarded as the most important diagnostic tool in this disease [for a review, see 38]. Nevertheless, chimerism analysis may be an important adjunct method to assess the response towards donor leukocyte infusion (DLI) [30, 32]. In addition, analysis of chimerism in bone marrow CD34+ cell before DLI may give valuable information on the risk of developing aplasia after DLI [39]. Chimerism analysis in transplant settings using T cell depletion to schedule T cell add-back and to monitor the success of this intervention may also be of special importance, especially in transplants with dose-reduced conditioning [40].

Conflicting data have been reported on the question of whether standard STR-based procedures are also useful for the early detection of reappearing disease in acute leukemias. There are several reports indicating that a level of about 1% may be sufficient to detect relapse in acute leukemia. Bader et al. [41] described the use of overall chimerism analysis in 30 transplanted children with acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) and MDS. Patients who showed mixed chimerism had a significantly higher probability of relapse compared to those with stable complete chimerism. However, there were also patients within this cohort who had increasing host chimerism without relapse as well as 3 patients who had complete chimerism before relapse. A later analysis by the same group of 55 patients confirmed these data [42]. Similar data, indicating that chimerism analysis from unsorted peripheral blood or bone marrow samples is able to detect relapse, were published by several groups [43, 44]. In contrast, other groups were not able to demonstrate a good prediction of relapse by analyzing chimerism from the peripheral blood. Suttorp et al. [9] showed that an RFLP assay with a 1% sensitivity showed complete chimerism 30–86 days before a clinical relapse was diagnosed. They concluded that the kinetics of reappearing leukemia are too rapid to be diagnosed with a detection limit of 1%. Comparable results were seen by other groups [45–48]. Taking these results together, mixed chimerism may precede subsequent relapse, but the interval between the decrease in donor cells using STR or VNTR-PCR and the clinical diagnosis is much too short to make meaningful clinical decisions in the majority of the patients. The

background of these discrepancies in the reports on the usefulness of chimerism analysis of peripheral blood is not yet clear. The reasons may be technical (e.g. different levels of sensitivity) or different sampling protocols. They may also be related to different patient populations, since the majority of data showing sufficient sensitivity are from pediatric transplantations. However, an increase in sensitivity is clearly desirable to improve the detection range of residual host cells and to potentially increase the time for clinical decision making.

New Techniques for the Detection of MRD Using Chimerism Analysis

As stated above, STR-based methods have a level of sensitivity which is in the range of 1–5%. One additional problem associated with this technique is the diagnostic uncertainty induced by PCR artifacts (stutter signals) [49], which renders a large number of peak constellations insufficient for analysis, because a residual host signal is located within the stutter peak of the donor, and thus cannot be detected accurately [50]. To overcome this limitation, a selection of STRs with a high proportion of donor/recipient constellations without interference of host and donor signals is desirable. In a recent analysis of 27 different STRs in a population of 203 HLA-matched related donors and recipients, we have identified a subset of markers with a high likelihood of finding such constellations [50a]. However, even if STR-based assays for chimerism analysis may be further improved and may detect leukemic cells in some patients, according to our experience and most of the published data the detection is too late in most of the patients to facilitate early intervention, e.g. tapering of immunosuppressive therapy. Thus in order to detect recurring disease as early as possible, novel assays with higher sensitivity would be a major improvement. Recently, quantitative chimerism analysis using real-time PCR has been reported for this purpose. Real-time PCR, either by the 5′-nuclease assay (TaqMan procedure) or by fluorescent resonance energy transfer, has the advantage that the product yield of the PCR is measured during each cycle, which allows the calculation of the number of starting molecules [for a review, see 22]. Real-time PCR monitoring has become a standard procedure for the evaluation of the response to therapy in acute and chronic leukemias and has been shown to be a sensitive and predictive tool for the guidance of treatment. It relies on the amplification of a specific target sequence, such as fusion oncogenes (e.g. bcr-abl, PML-RARalpha) or B or

T cell receptor rearrangements [22]. However, in a large proportion of acute leukemias, such specific molecular targets are missing. In male patients transplanted from a female donor, the Y chromosome represents a unique characteristic of recipient cells. Lo et al. [51] were the first to use real-time PCR for the SRY gene on the Y chromosome to detect microchimerism due to fetomaternal transfer in females who have given birth to male children. Fehse et al. [23] have reported a similar method for the evaluation of chimerism after hematopoietic stem cell transplantation (HSCT). However, clinical data on the use of this procedure are still missing. We used real-time PCR for the SRY gene on the Y chromosome to quantify the level of chimerism in 43 male patients transplanted from a female donor [52]. This method was shown to have a lower limit of detection of one male cell in 100,000 female cells. Our results indicate that residual host cells are detectable at very low levels in almost all patients investigated up to 5 years after transplantation. In addition, a gradual decrease of residual host cells was found over time (median 1 month: 0.3%; median 6 months: 0.01%; median 1 year: 0.0061%; median 4 years: 0.0015%). This persistence of host cells over long periods at low levels might explain why earlier reports using end point PCR did not show any impact of the detection of residual host cells [53]. In contrast, using real-time PCR, patients with relapse showed an increase in the percentage of host cells which preceded the haematological relapse by up to 120 days. Thus, real-time PCR might represent an interesting tool to analyze the level of residual host cells. As discussed earlier, the Y chromosome can be used only in a subset of patients. Several groups have now reported real-time PCR assays using other genetic targets, i.e. single nucleotide polymorphisms (SNPs), for the differential amplification of host cells after transplantation. SNPs are mostly biallelic genetic variances which occur on average every 1,000 bp, thus about 3,000,000 of these polymorphisms are present. Alizadeh et al. [54] recently published a set of 11 biallelic SNPs for real-time PCR analysis after allogeneic HSCT. These SNPs could discriminate 90% of the donor and recipient constellations tested. The assay has a minimum sensitivity of 0.1%, which is not as sensitive as for the SRY gene, but at least 10-fold better than for standard STR or VNTR-based methods. Maas et al. [25] also published data on the use of real-time PCR for chimerism analysis with very similar results. In summary, real-time PCR-based procedures will provide an important improvement of the diagnostic inventory for chimerism analysis. However, as shown by several authors, real-time PCR cannot totally replace STR- or VNTR-based

methods. One substantial drawback of this technique is the lower quantitative accuracy [25, 54]. This problem is inherent in the technique, since a change of only one PCR cycle means a 2-fold quantitative difference. This implies that a coefficient of variation of about 20–30% must be considered as normal. In contrast, STR-based procedures, especially when performed in a multiplex PCR, achieve reproducibility values between 4 and 8% [50, 55]. Thus in states of chimerism exceeding 5% host signal, STR analysis remains the method of choice. This technique is also superior to real time in the analysis of subset populations due to the limitations discussed above.

Another possibility to increase the sensitivity of a chimerism analysis is the use of specific subpopulations of the peripheral blood or bone marrow. The enrichment of cellular compartments has been shown to increase the sensitivity of chimerism analysis, since specific populations of cells, like B cells, T cells or early stem cells, are enriched from the background of mature granulocytes. Ginsburg and coworkers [56] were the first who described the investigation of cellular subsets after HSCT; van Leeuwen et al. [57] were the first to use highly purified cellular populations. Meanwhile, several groups have reported the analysis of chimerism in specific subsets to detect reappearing leukemic cells [27, 34, 58–61] or to monitor the effect of treatment [30].

Lamb et al. [58] described the use of subset analysis to differentiate suspicious relapse in two cases. In one patient they did not find evidence for relapse, whereas the second patient showed relapse in the CD34+/CD7+ leukemic cells, which was undetectable in the unsorted bone marrow material. Zetterquist et al. [60] compared chimerism analysis in sorted B cells with overall chimerism and PCR for clone specific T cell or B cell receptor rearrangements. Mixed chimerism in the B cell compartment was found in 5 patients who also showed persistence of the clonality marker in the PCR. Mixed chimerism in the B cells was detected 2.5 months prior to the morphological relapse in 3/4 patients with relapse. No relapse was observed in those seven cases with complete donor chimerism in the B cell compartment. Mattson et al. [34] studied 30 patients with AML and MDS. They used immunomagnetic labeling with antibodies against CD33, CD7 or CD45 to enrich the specific subpopulations from the peripheral blood or the bone marrow, achieving a final sensitivity between 2 and 4×10^{-4}. Mixed chimerism in these populations 1 month after transplantation was observed in 14 of the 30 patients. A relapse was seen in 10/14 patients with mixed chimerism in the subpopulations compared to only 2/16 cases with complete chimerism, a

difference which was highly significant. Interestingly, mixed chimerism in the peripheral blood seemed to have a lower sensitivity (67%), but a higher specificity (100%) in the prediction of relapse compared to the analysis of bone marrow samples.

We have recently performed a study using CD34+ cells as target for the identification of leukemic cells. The basic idea behind this was that the CD34 antigen is expressed on a very small population of normal hematopoietic progenitor cells, but can be frequently detected on blasts of different leukemias [62, 63]. Evaluation of this method showed a minimum sensitivity of 1/40,000 cells [33]. In a panel of 87 prospectively investigated patients, chimerism analysis in the CD34+ cells was performed for the detection of relapse [61]. After a median follow-up of 295 days (range 28–1,152 days), a total of 22 relapses were observed in the 84 patients showing engraftment. Relapse was associated with a decrease in CD34+ cell donor chimerism by up to 97 days in 20/22 patients. In patients with CML and molecular relapse, recurrent disease was associated with a decrease in donor CD34+ cells. Treatment with DLI or imatinib resulted in an increase in donor CD34+ cells and a clearance of bcr-abl-positive cells. Since this assay can be performed with peripheral blood, the investigations can be done at short intervals. These data inspired us to start a randomized prospective multicenter trial comparing chimerism from the peripheral blood and subset chimerism within the CD34 compartment in patients with AML, ALL or MDS whose leukemia blasts express the CD34 antigen. In patients showing a decrease in the proportion of donor CD34+ cells below 60%, immunosuppression is reduced or DLIs are given. First results of this study are inspiring and we have seen responses in several cases of AML and ALL.

Thus taken together, these data clearly show that subset analysis is a very sensitive technique, with a limit of detection comparable to nested PCR. This technique adds important information, since it is able to clarify whether reappearing host cells are of leukemic origin or are T cells or other nonmalignant cells.

In this article we have not focused specially on the use of chimerism in the setting of dose-reduced conditioning, since the detection of MRD is not substantially different after this form of transplantation. However, since mixed chimerism, especially in T cells, is much more common after dose-reduced preparative regimens, subset analysis is even more important to differentiate persistent mixed T cell chimerism from reappearing leukemic cells.

Use of Chimerism for Follow-Up after Transplantation: The Dresden Experience

Recently, several groups have published their procedures for the follow-up investigations after allogeneic HSCT [64–69]. These reports are mainly focused on technical issues. As discussed above these questions are certainly important. However, especially for the detection of MRD, the use of the appropriate methods according to the clinical situation is most important. Based on our own experience and the literature data, our current recommendations for chimerism analyses are as follows: During engraftment and during the entire period of mixed macrochimerism (i.e. chimerism between 3 and 97%), STR analysis, preferentially in a multiplex assay, should be used on whole blood or bone marrow samples to quantify chimerism. We usually perform these analyses twice weekly starting at day +5. If graft failure or relapse is clinically suspected, sorting of T cells, NK cells and myeloid cells, preferentially CD34+ cells, might be helpful. Once the chimerism in the peripheral blood has reached a level of more than 97% donor, real-time PCR assays should be used for further monitoring whenever possible. When real-time PCR indicates that the level of residual host cells further declines follow-up monitoring with real-time PCR is recommended at regular intervals. When this method indicates a level of residual host cells below 0.005%, the periods between the analyses may be increased to 2–3 months, because up to now we have not seen relapses when patients were at this low level. When real-time PCR assays cannot be used, standard STR-PCR should be performed at closer intervals. The length of the intervals should be chosen according to the tendency of relapse of the primary disease and the time after transplantation, with more frequent analyses (weekly to every 2 weeks) performed in patients with high risk disease (like AML or ALL) and early after transplantation. This very tight schedule should be followed for the first 2 years after transplantation, since the majority of relapses occur during this period. If real-time PCR shows that the level of host cells does not decline or increases again, subset analysis (including T cells, CD15+ cells and, if possible, CD34+ cells) should be performed in order to clarify the origin of these cells. Based on this information and the clinical situation (i.e. whether there is graft versus host disease present or not and the individual relapse risk), reduction of the immunosuppressive therapy or infusion of T cells might be performed.

Thus in conclusion, we believe that chimerism analysis can be performed for the detection of MRD, but the

diverse methods currently available differ in their abilities. If there is a genetic marker (translocation, IgVH rearrangement) which can be used for real-time PCR, this is certainly the first choice for MRD detection, since these markers are specific for leukemia. If not, chimerism analysis using the most sensitive and quantitative method available should be used. We believe that a strategy like this will allow for a more accurate and reliable assessment of chimerism and might help to identify patients at risk of a reappearance of leukemia. However, prospective trials will clearly have to show whether these strategies can be used to achieve a longer leukemia-free survival after transplantation.

Acknowledgments

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