Expert Review 08

Overcoming limitations in the detection of mixed chimerism

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About the author

Dr. Dan Hauzenberger is an associate professor and senior physician at Karolinska University Hospital, Sweden. He has held the position of Medical Director of the Section for Transplantation Immunology for 15 years.

For the last 20 years, he has had a focus on chimerism analysis in transplanted patients, and has led the development of new technology, including highly sensitive molecular diagnostics for chimerism analysis, at his laboratory. The Section of Transplantation Immunology at Karolinska University hospital has initiated the development of novel diagnostics in solid organ and stem cell transplantation and is one of the leading transplantation laboratories in this field in Scandinavia and Europe.

Introduction

The term chimera derives from Greek mythology and was first described by Homer in the Iliad as a fire-breathing monster in Asia Minor, composed from parts of multiple animals. Chimerism is defined as the presence of cells or tissues originating from another individual than the host derived cells.

This situation can occur naturally during pregnancy: where fetal cells circulate within the maternal blood stream, in dizygotic twin pregnancies with separate placentas, or after transplantation. The fact that cells from two (or more) genetically separate individuals can co-exist within one body has led to development of new techniques to discriminate the amount of the two genetic individuals within the organism.

While chimerism is to be expected with any transplantation, the amount of chimerism can differ in different transplant settings. For instance, solid organ transplantation means transferring tissues or solid organs into the host with little amounts of circulating donor cells within the blood stream. In contrast, hematopoetic stem cell transplantation (HSCT) results in continous recirculation of donor cells within the host. These facts have led to the evolution of different laboratory techniques to define the amount of donor DNA or cells within the host. One important reason for monitoring of patients post HSCT is to allow the earliest possible medical intervention and best possible patient outcomes.

This paper tries to highlight the importance of chimerism as a diagnostic tool for clinicians treating transplanted patients. Furthermore, the evolution of novel diagnostic tools for early detection of mixed chimersim is discussed. The recent development of NGS technology offers the possibility to analyse mixed chimerism with both sensitivity, as well as accurate and precise determination.

The importance of detecting mixed chimerism

The introduction of hematopoietic stem cells (HSC) from bone marrow (BM) or peripheral blood (PB) as a curative treatment for patients with malignant or non-malignant haematological diseases has been one of the major medical advancements of the last 30 years. In 1957 the first attempt at performing bone marrow transplantation was made in several patients suffering from malignant hematological diseases (Thomas, Lochte et al. 1957).

Early attempts at using hematopoietic allogeneic stem cell transplantation (HSCT) for treatment were however poor with many patients dying in complications directly related to the transplantation. However, with increasing knowledge of the importance of the polymorphic HLA system (Human Leucocyte Antigens) and immunosuppression, results of transplantations improved and are today the only curative treatment for patients with malignant or non-malignant haematological diseases (Ringden, Groth et al. 1995, Ringden, Lonnqvist et al. 1995).

Today more than 50 000 patients annually undergo HSCT world-wide. The majority of these patients have an underlying malignant disease such as acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), chronic myeloid leukemia in blast phase, Myelodysplastic Syndrome (MDS), multiple myeloma, high-risk lymphomas and Hodgkin's disease. Furthermore, several non-malignant diseases can also be treated successfully using HSCT. Among them are several immunodeficiencies, such as severe combined immunodeficiency (SCID), Wiskott-Aldrich syndrome, and common variable immunodeficiency (CVI) (Saba and Flaig 2002).

"...development of novel techniques for early detection of relapse has been of major importance for these patients"

At least four different clinical complications following HSCT have been described; toxicity related to the pre-treatment, infections, recurrence of the underlying malignant disease and immunological reactions including graft-versus-host-disease (GVHD) (Barrett, Horowitz et al. 1989, Horowitz, Gale et al. 1990).

Recurrence of the underlying malignant disease (relapse) is the most frequent cause of treatment failure in patients undergoing HSCT for leukaemia. Relapse rates of up to 20% have been reported in patients who received transplants in the early stages of their disease. Patients with more advanced diseases show higher incidence of relapse, reaching 50-70% in some reports, with T cell depletion and absence of GVHD as the most important risk factors (Horowitz, Gale et al. 1990, Marmont, Horowitz et al. 1991).

Leukemic relapse occurs, in general, in the recipient-derived cells due to incomplete eradication of the malignant clone, poor graft-versus-leukaemia (GVL) effect or de-novo malignant transformation following treatment with oncogenic substances. Treatment of malignant relapse can be performed in several ways including cyclosporine discontinuation, chemotherapy, second allograft, G-CSF or donor-lymphocyte infusions (Kolb and Bender-Gotze 1990). Therefore, in addition to treatment strategies for leukemic relapse, development of novel techniques for early detection of relapse has been of major importance for these patients.

The main aim of HSCT in patients with malignant diseases is, as mentioned, the eradication of the malignant cell clone. Since the complete eradication of the malignant cells is difficult to measure, complete remission (CR) has been used for defining successfully treated patients.

CR in acute leukaemias is in general defined as:

- Bone marrow blasts <5%;
- absence of circulating blasts;
- absence of extramedullary disease and recovering; or
- normalized peripheral blood counts.

"Success in treatment intervention is very much dependent on the availability of diagnostic techniques for early (i.e. sensitive) detection and quantification of minimal residual disease."

Determination of complete remission in patients undergoing HSCT has mainly been based on counting cells in blood and bone marrow using light microscopy or flow cytometry. The presence of minimal numbers of detected or non-detected malignant cells in blood or bone marrow has been termed minimal residual disease (MRD) (Lion, Daxberger et al. 2001, Uzunel, Jaksch et al. 2003). Success in treatment intervention is very much dependent on the availability of diagnostic techniques for early (i.e. sensitive) detection and quantification of minimal residual disease. Early treatment made possible by more sensitive diagnostic methods is expected to improve patient survival and significantly reduce the costs for management of transplant patients.

With the introduction of novel molecular as well as non-molecular techniques with higher sensitivity, the limit of detection of remaining malignant cell clones has vastly improved. An overview of the techniques available for detection of minimal residual disease and their limit of detection is shown in figure 1.

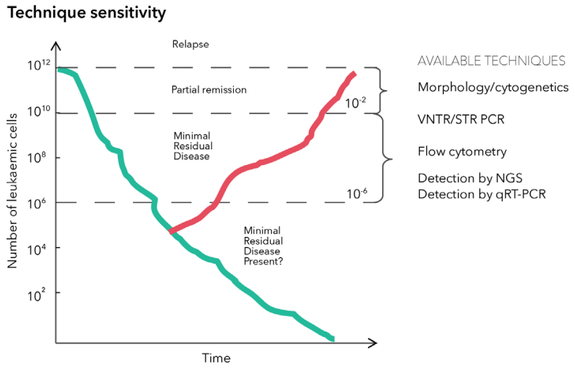

Fig. 1 Sensitivity and limit of detection of different techniques for determination of minimal residual disease.

Methods for detection of mixed chimerism (MC)

Flow cytometry
Flow cytometry was introduced in the 1960s and refers to an automated procedure in which a suspension of labelled as well as non-labelled cells flows past a light detector. The flow cytometer can thereby measure the number and size of the passing cells as well as cellular granularity. Furthermore, the method is well suited for detection of cell surface antigens using labelled antibodies.

Since flow cytometry is a sensitive method for detecting defined cell populations, it is currently used by many centres for the detection of malignant cells within a blood or bone marrow samples. The principle of this assay relies on the fact that leukaemia cells can display certain surface antigens which are not, or are poorly, expressed by normal cells. This is helpful in sorting out certain cellular subpopulations including malignant cells with a specific phenotype. Flow cytometry can detect a leukemic cell population if it represents more than 0,01% of the total investigated cell population.

However, although flow cytometry is well suited in defining certain cellular subpopulations including malignant cells, it is limited by identification of specific markers and clonal evolution which may cause false negative results. Therefore, new diagnostic methods based on molecular techniques have been developed to detect malignant cells present in low numbers.

Molecular techniques

1. RFLP
The first molecular technique used for chimerism analysis was restriction fragment length polymorphism (RFLP), which has a detection limit of 5-10 %.
The sensitivity of RFLP was further improved by using probes for variable number of tandem repeats (VNTRs).

2. PCR of Short Tandem Repeats
In order to increase throughput of sample numbers and to reduce hands-on time, quantitative PCR was used to analyse chimerism in patients undergoing HSCT.

For instance, Y-specific PCR which improved sensitivity to 0,01%, was used to follow HSC transplanted patients (Landman-Parker, Socie et al. 1994, Petit, Raynal et al. 1994). An important drawback of this technique was its restriction to sex-mismatched transplantation situations.

PCR of short-tandem repeats (STR) do not have the above-mentioned restrictions and is currently the method of choice for many laboratories. An STR is a pattern of two or more nucleotides that are repeated directly adjacent to each other. The role of these repetitive units (satellites) is not well understood but more than 50 000 satellites are estimated to exist within the human genome. The fact that STRs present high levels of inter- and intra-specific polymorphism have made them suitable targets in for forensic and paternity investigations.

Analysis of STRs using semi-quantitative PCR methods is based on the PCR amplification, post-PCR separation and identification of of STR repeats in DNA from clinical samples. The relative allele dosage of recipient- and donor-derived STRs is subsequently determined (see figure 2). The technique has a limit of detection of the minor allele at a level of 1-5 %. One drawback is that the technique is semi-quantitative and thus the results are dependent on the relative amounts of the separate alleles and results can vary depending on which of the alleles is shorter (shorter DNA sequences are generally more efficiently amplified in PCR than longer DNA sequences).

3. qPCR
New techniques for rapid detection and identification of unique DNA sequences have met increasing interest for use in diagnostic areas outside of the transplantation field. One such technique, quantitative real-time PCR (qPCR), is widely used for identification and quantification of DNA targets within unknown samples, such as viral DNA detection and quantification (Boissinot and Bergeron 2002, Niesters 2002).

qPCR is a true quantitative technique which monitors the fluorescence emitted during the reaction as an indicator of target amplification during each PCR cycle (i.e., in real time) as opposed to the endpoint detection provided by conventional PCR methods including STR-detection. By recording the amount of fluorescence emitted during each cycle, it is possible to monitor the PCR reaction during the exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of the target template. The earlier a signal is detected, using this technique, the higher amount of target DNA is present in a sample (figure 2).

qPCR protocols for the detection of mixed chimerism are based on discrimination of the inter-individual variability of single-nucleotide polymorphisms (SNP) or different insertion-deletions (indels) between two individuals. qPCR has been demonstrated to be 100 times more sensitive compared to semi-quantitative methods (Alizadeh, Bernard et al. 2002). When used for detection of mixed chimerism in a subset of acute leukaemias, relapse can be detected up to 6 months earlier as compared to STR-analysis. This level of sensitivity increases the possibility of early clinical intervention and thus increases the chance of a positive transplantation outcome.

"STR based techniques, in general, show good precision but a limited LOD (limit of detection) whereas Q-PCR exhibits poor precision, especially at higher amounts of mixed chimerism, despite good LOD."

Although both the STR and qPCR methods have clear advantages there are also some disadvantages with these techniques. The STR based techniques, in general, show good precision but a (narrow or constrained) limit of detection (LOD) whereas qPCR exhibits poor precision, especially at higher amounts of mixed chimerism, despite good LOD. It has therefore been of interest to develop novel methods which combine the advantages of STR and real-time PCR without their respective draw-backs.

4. NGS

One technological platform which may fulfil the above-mentioned requirements is Next-Generation Sequencing (NGS). NGS, also known as massively parallel sequencing, describes several different modern sequencing technologies which have allowed the sequencing of DNA and RNA in parallel and on a massive scale. NGS has as such revolutionized the study of genomics and molecular biology at large scale (Behjati and Tarpey 2013).

There are a number of different NGS platforms which have in common the fact that they perform clonal sequencing of millions of small DNA fragments in parallel and subsequently compare these fragments with reference sequences using different advanced bioinformatic software tools (van Dijk, Auger et al. 2014),(Behjati and Tarpey 2013). This technique has thus given us the ability to perform whole-genome sequencing and whole-exome sequencing as well as targeted sequencing of selected genes or gene groups (van Dijk, Auger et al. 2014). NGS applications have been developed for: de novo sequencing of bacterial and viral genomes searching for genetic variants by resequencing whole genome or targeted genome regions, sequencing the transcriptomes of cells, tissues and organisms, and sequencing of epigenetic markers such as genome-wide profiling of DNA-binding proteins and epigenetic markers by ChIP-Seq among others (Cui, Dhroso et al. 2015).

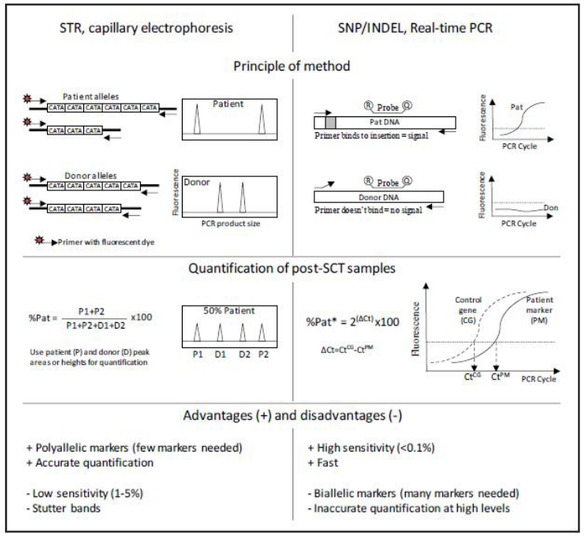

Fig. 2 Principle of chimerism analysis using STR and Q-PCR techniques (adopted from Uzunel et al.)

Recent advancements

Although several NGS assays for qualitative analysis of the genome or selected genes have been developed, few NGS-based tests for quantitation of DNA sequences in samples have been developed. Recently, novel NGS-based methods for chimerism analysis have been presented (Aloisio et al. 2016),(Pettersson, L. et al. 2019, manuscript in preparation). One of these assays, which is based on quantitative detection of a selected number of markers distributed throughput the human genome and shows sensitivity down to 0,1% LOD as well as good accuracy over a wide dynamic range. (Pettersson, L. et al. 2019, manuscript in preparation). This data indicates that NGS platforms may be suitable to develop assays that exhibits all the advantages of STR and Q-PCR for chimerism analysis without the previously described drawbacks.

One further improvement of the sensitivity of chimerism analysis following HSCT has been the lineage-specific chimerism in blood and bone marrow. As far back as 1995, Socie et al had already suggested that in order to understand the dynamics of engraftment following HSCT, specific cell subsets should be investigated (Socie, Lawler et al. 1995). Since then, several studies have shown that lineage-specific analysis is important when investigating the kinetics of engraftment after HSCT (Roux, Helg et al. 1992, Roux, Abdi et al. 1993, Roux, Helg et al. 1996, Zetterquist, Mattsson et al. 2000, Mattsson, Uzunel et al. 2001). Lineage-specific chimerism analysis has helped to further increase the sensitivity of the tests. For instance, in acute B-lymphoblastic leukaemias (B-ALL), which represents one of the most frequent malignant disorders of childhood, lineage-specific chimerism improved the time between first detection of recipient-derived B-cells and clinical relapse by several weeks.

The median time between first detection of recipient-derived B-cells (>0,1 % in peripheral blood) and clinical relapse was 5.5 months whereas the median time between first detection of recipient-derived B-cells (>1 % in bone marrow) and relapse was 18 months (Uzunel, personal communication).

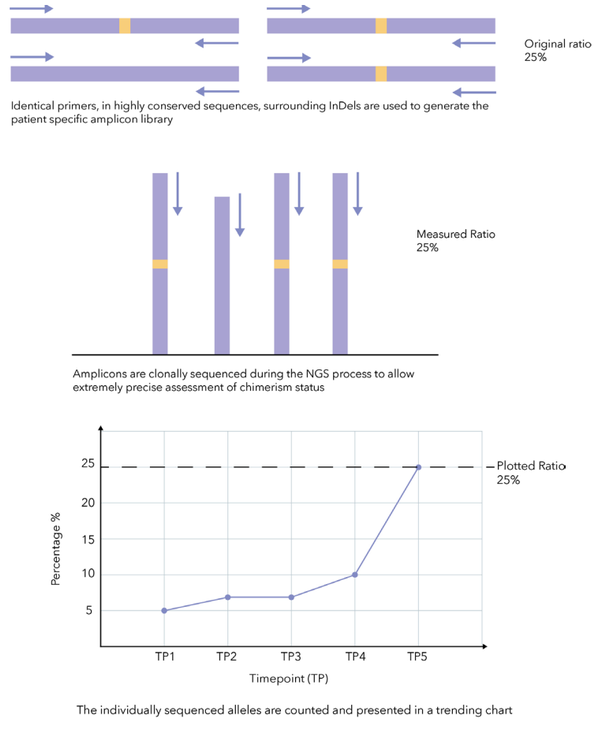

Fig. 3 Principle of chimerism analysis using NGS

Conclusion

The field of hematopoietic stem-cell transplantation has been improved by the development and implementation of methods which can detect mixed chimerism on a molecular level. The use of highly-sensitive techniques, such as NGS based assays, in combination with lineage-specific DNA enrichment, represent state-of-the-art techniques for chimerism analysis following HSCT.

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