Using PCR for molecular monitoring of post-transplantation chimerism

Utilização da técnica de PCR para monitoramento molecular de quimerismo pós-transplantado

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ABSTRACT

Post-transplantation monitoring has become a major tool for improving graft outcome, and the term chimerism is currently used for describing the presence of donor cells in transplant recipient patients. The present article describes different applications and advances of the methodologies used for detection and quantitative monitoring of post-transplantation chimerism, especially the PCR methodology using fluorescent primers and automated sequencers for amplification and detection of STR markers.

Keywords: Chimerism; Transplantation, Polymerase chain reaction; Genetic markers

RESUMO

O monitoramento pós-transplante de órgãos sólidos e medula óssea tornou-se uma ferramenta imprescindível para aumentar a sobrevida dos enxertos, sendo o termo quimerismo utilizado para descrever a presença de células do doador nos pacientes receptores de transplantes. O presente artigo descreve as diversas aplicações e avanços nas técnicas que podem ser utilizadas para a detecção e monitoramento quantitativo do quimerismo pós-transplantes, em especial a metodologia de PCR utilizando oligonucleotídeos fluorescentes e sequenciadores automáticos para amplificação e detecção de marcadores conhecidos como microssatélites, ou STRs.

Descritores: Quimerismo; Transplante; Reação em cadeia da polimerase; Marcadores genéticos

Various aspects, such as advances in immunosuppression and broader knowledge of immunology, better immunogenetic selection of donors and reduced mortality secondary to infection, have contributed to the success of transplantation as a therapeutic modality in patients with terminal dysfunctions, oncohematologic or congenital diseases or immunodeficiencies.

Post-transplantation monitoring of solid organs and bone marrow has become a major tool for improving the outcome of grafts, and the term chimerism has been used to describe the presence of donor cells in transplant recipient patients. A significant advance in this monitoring was achieved with the development of quantitative analyses, that is, the assessment of the proportion of donor and recipient cells present in the transplanted patient. This quantification is crucial for the monitoring of donor cell engraftment, for determining recurrence of the original disease after several types of transplantations, and it enables monitoring of the graft at different intervals of time after transplant(1). Moreover, this information is important to establish treatment strategies which may induce a stable chimerism.

The objective of hematopoietic progenitor cell (HPC) infusions is to repopulate the bone marrow after using myelosuppressor or myeloablative therapy. The sources for obtaining HPC are bone marrow, peripheral blood collected by apheresis after blood precursor cell mobilization, and cord blood(2).

In bone marrow transplantations, it is expected that the donor’s HPC recompose the patient’s bone marrow, establishing a complete chimera. Quantification of the proportion between the donor and the recipient cells allows a routine evaluation of the transplant engraftment and of the existence of total or mixed chimerism. Total chimerism occurs when the transplant recipient’s entire bone marrow is of donor origin, whereas mixed chimerism means that recipient cells are also present.

In non-myeloablative transplantations, the progression from mixed to complete chimera has to be monitored. There are, however, several other applications for the chimerism tests in bone marrow transplantations, as follows:

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Received on March 14, 2006 – Accepted on March 29, 2006
1. To evaluate the persistence of donor cells in patients with inadequate bone marrow function; in patients who are candidates for donor lymphocyte infusion or for a second allogeneic transplantation; and in the long-term follow-up of patients at high risk of rejection;
2. To determine the risk of graft versus host disease (GVHD), rejection or recurrent malignant disease;
3. To define whether recurrent malignancy originated from donor or from recipient cells;
4. To identify the presence of donor cells in pre-transplantation patients with severe congenital combined immunodeficiency, since usually children with this syndrome have occult maternal T-cells in their circulation. The presence of such cells requires immunosuppression to prevent rejection;
5. To correlate the immune recomposition after transplantation for the treatment of severe combined immunodeficiency;
6. To determine whether the cells of a transfusion donor are implied in the onset of GVHD. This is rare, but can occur due to the use of non-irradiated transfusion products. It can be demonstrated by the presence of genetic markers different from both the donor and the recipient;
7. To assess the genetic identity of twins, since with an identical donor there is no risk of rejection or GVHD, so there is no need for immunosuppressor treatment before or after the transplant.

GVHD can occur not only in transplants for oncohematologic diseases, but also in cases of solid organ transplantations, as for example, liver transplantation. GVHD is a major cause of non-diagnosed death in this kind of transplant, occurring in approximately 1% of all cases. At the onset of its manifestations, it is very difficult to be distinguished from viral infections or drug-related reactions, as the patients develop clinical pictures of fever, diarrhea, skin rash or pancytopenia, two to six weeks after transplantation. One of the most important risk factors for the development of GVHD after liver transplant is a high degree of HLA compatibility between the donor and the recipient, mainly for HLA antigens of loci A and B. This problem has already been described in lung and kidney transplantations.

Several methods were described for the analysis of chimerism. The genetic markers and laboratory methods used to monitor chimerism have progressed considerably along the history of transplantation since the early studies which used red cell antigens to prove the presence of donor cells after transplantation. Currently, there are several sensitive methodologies available to determine the status of chimerism, such as: HLA typing, cytogenetics and fluorescence in situ hybridization (FISH), polymerase chain reaction (PCR) to amplify repetitive DNA regions (VNTR's – variable number tandem repeats and STR's – short tandem repeats). The FISH technique is particularly well-established but, although it is rather sensitive and quantitative, it is limited to the cases in which the donor and the recipient are of different genders, since the chimerism marker is the sex chromosome. Class I HLA typing of patients is another rather useful methodology, but it can only be used when there is an HLA mismatch between donor and recipient. Moreover, it does not provide quantitative results.

In the 1980's, the molecular biology techniques started a revolution in the use of genetic markers, and the first of them to be used was the VNTR analysis technique. By this technique, the inherited DNA polymorphisms are detected using Southern blot and hybridization with specific probes. This method has not been used too much to determine chimerism because it is rather complex, very time-consuming and has poor sensitivity (around 10%), requiring the use of radioactive probes. In addition, the result does not allow a precise quantification.

Recently, several authors have demonstrated a variety of applications for the PCR technique using fluorescent oligonucleotides and automated sequencers for amplification of markers, known as microsatellites or STR's. STR's are regions of the human genome DNA composed of repetitions of small sequences of 2 to 6 nucleotides (for example, “AT” which appear a variable number of times (i.e., ATATATAT = (AT)6). The number of repeats at each locus is an inherited characteristic and can be used to identify the DNA of each individual. There are characterized STR loci on each human chromosome, and the use of multiple loci – at least 13 different loci – can identify an individual with fairly good certainty, making it therefore a highly informative methodology for identity, paternity and forensic tests. Moreover, in transplantations, this methodology allows a relatively quick quantitative differentiation of recipient and donor cells. The principle of STR analysis at one locus can be seen in figure 1.

The advantages of STR analysis over other methods are:
1. A very small quantity of DNA (nanograms) is sufficient for this analysis due to previous amplification by PCR;
2. It is a very sensitive assay, as it can distinguish the donor DNA from the recipient DNA even at percentages of 1 to 5%;
3. Informative results are obtained in the majority of analyzed cases.
4. The proportion between the obtained products can be quantified by computer analysis.

To determine the genetic profiles of patient and donor, samples of their DNA’s have to be tested. The best samples from the patient are obtained prior to transplantation, but if such samples are not available, hair root DNA can be used. As for the donor, oral mucosa or blood samples can be collected, or even histological samples stored in paraffin can be used. The result of the pre-transplantation sample profiles is used to select STR loci which are informative (in other words, that have different alleles in the recipient and in the donor) and will be tested in the post-transplant samples. The probability of finding informative markers depends on the number of loci tested, on the number of alleles of each locus, on the distribution of the alleles, and on the degree of relatedness between donor and recipient.

The STR results can even be used to quantify the contribution of donor cells in different specific cell lines of the recipient, separated by monoclonal antibodies (for example, CD3 and CD8 = T cells and suppressor T lymphocytes, CD20 = B cells, CD15 = mature myeloid cells) attached to magnetic particles or by a flow cytometer and cell sorter.

The proportion between donor and recipient DNA can be measured by comparing the relative difference between the heights of the peaks of each allele in the profile of each STR locus, using automated sequencers and multiplex PCR for analysis of several regions at the same time.

For the calculation, the values are used in relative fluorescence units (RFU), generated by the sequencer and corresponding to the size of the peaks of each allele of the recipient (R) and of the donor (D), and the following formula:

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\% \text{ chimerism} = \frac{\text{Allele}_1 \text{R} + \text{Allele}_2 \text{R}}{\text{Allele}_1 \text{R} + \text{Allele}_2 \text{R} + \text{Allele}_1 \text{D} + \text{Allele}_2 \text{D}} \times 100
\]

where R represents the value in RFU of the recipient’s peak height and D of the donor’s.

In those cases in which one allele is lost, the calculation is adjusted so as to reflect equal numbers of alleles for both the donor and the recipient, in order to prevent an incorrect result. Moreover, a mean is done for the alleles of the component (D or R) that possesses the two alleles. In these cases:

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\% \text{ chimerism} = \frac{\text{Allele}_1 \text{R}}{\text{Allele}_1 \text{R} + [(\text{Allele}_1 \text{D} + \text{Allele}_2 \text{D})/2]} \times 100
\]
The percentage of chimerism is determined for each informative marker, and the mean of the results of all markers (at least two) is used to generate the final chimerism result.

A few examples of different results obtained using STR’s can be found in figure 2.

In conclusion, the use of the PCR technique for the amplification of microsatellites marked with fluorescent oligonucleotides to monitor total or mixed chimerism has shown to be fast, sensitive and reliable, besides presenting results which are consistent with the clinical and hematological data. Several authors have demonstrated its application in different types of transplantations, and this technique has been widely accepted in the follow-up of patients after allogeneic bone marrow transplantation. Moreover, the possibility of using commercial kits and computer analysis programs already available on the market for human genetic identification makes the method easier to standardize by laboratories which are interested in setting up this new tool for making therapeutic decisions.

The Laboratory for Special Techniques of the Clinical Pathology Department of Hospital Israelita Albert Einstein, with its experienced team in diagnostic molecular biology, human identification tests, analysis and typing of polymorphisms in human genetics and histocompatibility, has standardized a methodology that allows the simultaneous analysis of 15 polymorphic STR loci, besides sex chromosomes X and Y. For this purpose, genetic identification kits are used and analysis is made with a recently acquired automated nucleic
acid sequencer. The objective of setting up this technique was to contribute to the transplantation services, allowing a precise identification and follow-up of post-transplantation chimerism cases.

REFERENCES