

## MINIMAL RESIDUAL DISEASE (MRD) IN LEUKEMIA

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**Abstract:** Resistance to therapeutic agents is a main factor in the failure of cancer treatments. In leukemia, the resistant cells remaining in the bone marrow or peripheral blood constitute minimal residual disease and can be detected by highly sensitive assays when the patient appears to be in complete remission. Currently three different techniques with sensitivity of at least  $10^{-3}$  (one leukemic cell between  $10^3$  normal cells) are used for MRD detection: flow cytometric immunophenotyping, which is based on the detection of abnormal or unusual phenotypes; PCR analysis of patient-specific junctional regions or rearranged immunoglobulin (Ig) or T cell receptor (TCR) genes and PCR analysis of break-point fusion regions of chromosomal aberrations. Studies of MRD provide means of detecting relapse at sub-clinical levels and permit early intervention, this method being, therefore, highly useful in improving the oncology patient's clinical management. This paper aims to present, from a large body of data among the patients with leukemia, the current state and development of molecular techniques in the growing field of emerging methods that can detect MRD.

### INTRODUCTION

Acute leukemia includes a heterogeneous group of neoplastic disorders with great variability in clinical course and response to therapy, as well as in the genetic and molecular basis of the pathology. In these types of aggressive cancers, the bone marrow makes a large number of abnormal white blood cells that are not fully developed.

Minimal or submicroscopic residual disease (MRD) represents a small number of leukemic cells that remain in the patient's body during treatment or after treatment when the patient is in remission. This is the major cause of relapse in cancer and especially leukemia, thus studies during treatment hold great potential for improving the clinical management of patients with acute leukemia.

Cancer cells are the result of a single malignantly transformed cell, therefore these cells are clonally related. Hence, monoclonality is a key feature of malignant tumor cell populations, which allows discrimination between oligo or monoclonal and polyclonal reactive processes (Langerak et al, 2007). Clonality assessment and detection is possible via several technical approaches such as the study of chromosomes, DNA markers, tumor specific proteins and patterns of proteins known as tumor phenotypes.

One of the many different markers that can be used for clonality testing in suspected lymphoproliferations is the immunoglobulin (Ig) and T-cell receptor (TCR) antigen receptor gene rearrangements (Langerak et al, 2012). These Ig and TCR rearrangements occur in developing lymphocytes during the early stages of T and B cell maturation through somatic V(D)J (V-variable, D-diversity and J-joining) recombination and results in the highly various repertoire of antibodies/immunoglobulins (Igs) and T cell receptors (TCRs) found in B cells and T cells, respectively (Owen et al, 2013).

The most widely currently applied MRD assays in acute leukemia are flow cytometric identification of aberrant immunophenotypes and polymerase chain reaction (PCR) amplification of fusion transcripts and rearranged antigen receptor genes (Neale et al, 2004).

MRD assays can accurately measure treatment response and allow estimates of the residual leukemic cell load during clinical remission in individual patients, thus improving the selection of therapeutic strategies and, potentially, long-term clinical outcome.

### CYTOGENETIC AND MOLECULAR ANALYSIS OF MINIMAL RESIDUAL DISEASE IN LEUKEMIA

In the last decade the rapid progress in understanding the etiology of leukemic malignancies and technological advances has increased the specificity and sensitivity of detection of cancer cells at patients who appeared to be cured or in remission by conventional techniques (Cross 1997). Therefore, the therapeutic response of the patient can now be assessed by monitoring minimal residual disease (MRD) which means the detection of malignant cells at  $\geq 1 \times 10^{-4}$  sensitivity, at subclinical levels.

For a long period of time, clinicians relied on examination of cellular morphology in peripheral blood and marrow collected at regular intervals from asymptomatic patients in order to observe the patient's long-term response to therapy. These studies were and remain important but they lack sensitivity, as a consequence, the patients frequently had advanced disease that was difficult to treat by the time that relaps was detected (Kaeda et al, 2002).

### *Cytogenetic techniques*

By means of morphological techniques in leukemias and non-Hodgkin lymphomas the detection limit for the identification of reduced number of malignant cells is not lower than 1% (1 malignant cell among 100 normal cells). Therefore, van Dongen et al (1986) proposed different techniques for the detection of small numbers of cancer cells such as cytogenetics, cell culture systems, premature chromosome condensation and recombinant DNA techniques. Unfortunately, most of these techniques do not lower the 1% detection limit.

Acute leukemia is generally considered to be in remission when cancer cells reach less than 5% of the bone marrow cell population; however, Ryan and van Dongen (1988) demonstrated that patients with acute leukemia may have approximately  $10^{12}$  malignant cells at diagnosis and those in remission by this criterion may have as much as  $10^{10}$  undetectable neoplastic cells.

Conventional karyotyping based on chromosomal abnormalities that were observed at diagnosis has been used to monitor residual disease. The main advantage of this method is that it permits clear identification of the leukemic cells. In early studies of patients with acute myeloid leukemia (AML) the disappearance of the abnormal karyotype is generally correlated with the clinical remission as Testa et al (1979) observed in a study of patients with acute myeloid leukemia. They did not detect abnormal cells in patients in remission, although single abnormal cells of clonal origin could occasionally be observed. Hart et al (1971) reported the same observation in a study involving 10 patients with chronic myeloid leukemia (CML); each of the patients who relapsed had the same karyotypic abnormality that was found at diagnosis. More recently, Freireich et al (1992) reported that abnormal metaphases, also identical to those found at diagnosis, were observed at 20 of 71 patients with acute myeloid leukemia in morphologic remission. All 20 patients relapsed in the next period of time, thus maintaining the reliability of positive karyotypic analysis as a predictor of eventual relapse. Nevertheless, a number of 25 patients within the 51 patients with negative findings also relapsed, indicating that failure to detect abnormal clones does not necessary guarantee durable remissions. Therefore, karyotypic changes can have prognostic significance which may be useful in decisions on the aggressiveness of further therapy.

Metaphase analysis by conventional banding techniques is a laborious procedure with a success rate depending on the number of metaphases that can be observed and on the proliferative rate of leukemic cells that varies from case to case. Thus, there has been considerable effort to develop techniques that would ease metaphase screening.

*Fluorescence in situ hybridization (FISH)* techniques are based on chromosome-specific and gene specific DNA probes to identify numeric and structural chromosomal abnormalities. FISH can be combined with morphologic analysis to amend the accuracy of the results (Pinkel et al, 1988). The main advantage of this technique is that it provides interpretable information with use of nondividing cells, raising the chances of identifying abnormalities among the cells having a reduced proliferative rate.

The sensitivity of FISH technique was well demonstrated by research of Heerma et al (1993) who used probes of chromosomes X, 10, 17 and 18 to observe early remission in patients with acute lymphoid leukemia (ALL). The mean number of aneuploid cells observed in three normal

bone marrow samples was 8 of 2000, and one month after diagnosis, tri an tetrasomic interphases increased significantly compared to control values in three of the seven patients studied; also pentasomy and hexasomy that were not found in control samples were observed in five of the seven cases studied.

Nylund et al (1994) used FISH to detect numeric chromosomal abnormalities in interphase and metaphase cells and targeted translocations in metaphases cells in patients with various hematologic malignancies. From seven patients with AML, three had cells with abnormal karyotype in morphologic remission bone marrow samples, two of whom relapsed. By contrast, none of the FISH-negative remission samples patients had a recurrence. The conclusion was that FISH analysis of remission in ALL was less informative and although no abnormal karyotypes were detected in remission bone marrow samples of five patients, two relapsed.

The limitation to the presence of aneuploid but not leukemic cells makes the sensitivity of MRD analysis by FISH approaches only 1% (Gray et al, 1990). Methods to detect structural chromosomal abnormalities in interphase cells by FISH with locus-specific probes have also been developed. A well-known example is *BCR* and *ABL* genes labeled with different fluorochromes used to identify the t (9;22) translocations.

In the 1980s, main approach to assess the response to treatment was repeated bone marrow metaphases analysis for the presence of the Philadelphia (Ph) chromosome in patients. Bartram et al (1983) described initially the application of in situ hybridization for detection of the translocation of *ABL* to the Ph chromosome. In around 90% of cases of chronic myeloid leukemia, chromosomal material is reciprocally exchanged between the long arm of one chromosome 9 and the long arm of chromosome 22, translocation referred to as t(9;22)(q34;q11). The derivative 22q- is the Ph chromosome. The hallmark of chronic myeloid leukemia (CML) is the formation of a *BCR-ABL* fusion gene, usually as a result of the Ph chromosome translocation. Melo et al (1993) showed that the *ABL-BCR* gene formed on the derivative chromosome 9q+ is transcriptionally active in 65% of the CML patients involved in their study. However, the sensitivity of this approach may have limitations because in about 5% of normal lymphocytes artifactual colocalization may appear (Arnoldus et al, 1990).

### ***Flow-cytometry***

Some groups have proposed the use of flow-cytometry as mean to detect chromosomal abnormalities, having a higher accuracy than conventional banding techniques. The first approach to investigate the applicability of this technique was done by Arkesteijin et al, in which they used a two-colour analysis with chromomycin A3 (which labels GC base pairs) and Hoechst 33258 (which labels AT base pairs), procedure that resolved all chromosomes except numbers 9 to 12. The analysis revealed the percentage of subclones containing a certain chromosome anomaly, confirmed by the conventional cytogenetic analysis. Although it is not possible by this technique to determine the position of the breakpoint, the involved chromosomes in the translocation event could be identified, but also, in some cases, the low percentages of aberrations could not be detected. This study showed that CML can be diagnosed on the basis of flow karyotypic results and additional chromosomal aberrations can be detected provided that changes in the amount of DNA per chromosome have occurred. The precise quantification of the composition of subclones in the case of mosaicism appears difficult.

Another method of monitoring MRD relies on the identification of aneuploidy by single-laser cytometry in cells labeled with DNA-binding fluorochromes such as propidium iodide and 7-actinomycin D (Rabinovitch et al, 1986). Pantazis et al (1987) observed the course of a patient with

AML reporting that the disappearance of aneuploid peaks in flow cytometry coincided with morphologic remission and Redner et al (1990) detected early relapse in a patient with acute lymphoid leukemia that was in clinical remission.

Nevertheless, flow-cytometry has some specific limitations. Extreme sensitivity, such as detection of 1 leukemic cell among  $10^5$  or more normal cells is difficult to achieve by flow-cytometry and such high sensitivity is important in studies deeking MRD in patients. Another limitation is that the immunophenotype of leukemic cells may change during the progression of the disease, which may conduct to a false-negative result (Baer et al, 2001).

### ***Molecular studies***

Though cytogenetic analysis and FISH studies remain extremely valuable in the initial investigation of malignant hematopoietic disorders, their role in monitoring MRD has decreased with the introduction of molecular techniques.

The PCR technique allows the amplification of tumor-specific DNA sequences or mRNA sequences (after reverse transcription into cDNA), if the flanking sequences are well defined. This PCR-mediated amplification can detect specific sequences derived from only a few cancer cells among normal cells. Well-defined chromosome translocations such as t(9;22) have been used as tumor-specific markers (van Dongen et al, 1991). The advantage of using specific chromosome aberrations as tumor-specific markers is their stability during the progress of the disease. Whether genomic DNA or cDNA obtained from RNA is used in this procedure depends on the molecular target, this method having an extremely high sensitivity for detecting kariotypic abnormalities. Experiments with artificial mixtures of leukemic and normal cells were conducted by Cross et al (1993) and have consistently shown detection of a single leukemic cell among  $10^5$  to  $10^6$  cells.

Among the leukemic lymphoblast-specific fusion transcripts that lately became targets of PCR analysis, only those connected with the Ph chromosome have been repeatedly applied in the study of MRD (Miyamura et al, 1992). Miller et al. (1993) showed that serial negative results obtained using PCR were correlated with prolonged disease-free survival of the patient, whereas one or more positive tests after treatment were associated with subsequent relapse.

The RT-PCR has been widely exploited to detect the different *BCR-ABL* transcripts by multiplex PCR (which has a sensitivity of  $10^{-2}$  –  $10^{-3}$ ) and has enhanced the level of sensitivity of MRD detection. A sensitivity of  $10^{-5}$  –  $10^{-6}$  is achievable by nested PCR in a clinical laboratory (Biernaux et al, 1995); in these conditions *BCL-ABL* mRNA was detected in a high proportion of normal healthy individuals (Bose et al, 1998). A sensitivity of  $1 \times 10^{-5}$  is achievable by Q-PCR, but a major concern is contamination and false-negative results due to the lack of mRNA or sub-optimum integrity of mRNA/cDNA (Beillard et al, 2003).

## **DETECTION OF ANTIGEN-RECEPTOR GENE REARRANGEMENTS**

The antigen-receptor genes include several discontinuous germline segments (V-variable, D-diversity and J-joining) that undergo clonal rearrangements in lymphoid cells. Analysis of Ig and T-cell receptor (TCR) gene configurations can be used to monitor the persistence of malignant clones whose rearrangements were determined at diagnosis. The B and T-clonal recombinations generate patient-specific DNA length and sequences which are ideal molecular markers for detection and quantification of leukemic cells among normal lymphocytes in remission samples. Although sensitive, this technology is susceptible to false-negative results due to clonal evolution during the progression of the disease, thus some patients may relapse with a clone different to the

one observed at diagnosis. The risk of false-negatives can be diminished by targeting two Ig/TCR gene rearrangements when conducting MRD-PCR studies (Van der velden et al, 2008).

The sensitivity of PCR for antigen-receptor gene rearrangements varies with the uniqueness of the leukemia-specific regions of the genes (Bregni et al, 1989). In a study conducted by Brisco et al (1994) PCR amplification of IgH genes followed by hybridization with clonospecific probes lead to a detection level of 1 leukemic cell in  $10^4$  or fewer normal cells in 42 of 88 cases. Similarly, in 71 cases studied by Bartram et al (1993) with PCR amplification of TCR genes, the detection level reached 1 leukemic cell in  $10^4$  or fewer normal cells in 33 cases; a sensitivity of 1 in  $10^5$  was achieved in 29 cases, whereas 1 in  $10^6$  was obtained in only 9 of the 71 cases.

Specific primers for individual V and J regions or consensus primers for conserved regions can be designed. For example, the approximately 100 VH genes can be grouped into seven different families with homologous sequences (Stewart et al, 1994). The most conserved regions known as framework regions and the regions that encode the antigen-binding site of the Ig heavy chains are also known as complementarity-determining regions (CDRs). The CDRs that are encoded by the VH gene region are CDR1 and CDR2; by contrast, the CDR3 region comprises in the 3' end of VH, all of D and 5' end of JH, and the N nucleotides assembled during the recombination process. This region is specific to each lymphoid clone. To amplify the rearranged IgH genes, a consensus JH primer, a panel of VH primers specific to VH families can be used (Deane et al, 1990), thus detecting the Ig gene rearrangements in 90% of the cases of B-lineage ALL cases at diagnosis as Deane et al, reported with a 75% success rate.

In T-lineage ALL the issue that appears is that IgH rearrangements are usually incomplete. TCR $\alpha$  and TCR $\beta$  genes have a large number of functional V and J segments whereas TCR $\gamma$  and TCR $\delta$  contain only a few (Davis et al, 1988), thus the potential for combinatorial diversity is higher. Efforts to determine rearranged TCR $\gamma$  genes using consensus primers were successful in approximately 90% of the cases (D'Auriol et al, 1989).

Nevertheless, in contrast with abnormal gene configurations caused by chromosomal translocations, the detection of a PCR-amplified signal from antigen-receptor genes cannot be taken as evidence of MRD until the signal is differentially distinguished from the background, originating from normal lymphoid cells. This can be resolved by amplifying the clonal IgH rearrangements with single VH family-specific primers, then identifying them on the basis of size and signal intensity after separation by high-resolution gel electrophoresis (Cole-Sinclair et al, 1993). The electrophoretic profiles obtained with remission samples are compared with the diagnostic DNA for the presence of similar dominant bands. Clonally rearranged Ig and TCR genes can be observed by analyzing the junctional regions TCR $\gamma$  or TCR $\delta$  genes or the CDR3 regions of the IgH genes. Similar to PCR amplification of translocation breakpoints, the leukemia-specific primers detect PCR-amplified signals only in the presence of the malignant clone. Beishuizen et al (1994) detected bi or oligoclonal IgH rearrangements at diagnosis in 8 of 30 cases of B-lineage ALL and also noted differences in the rearrangement of patterns in 20 of the cases at the time of relapse. They observed a correlation between shifts in antigen-receptor gene rearrangement pattern and the duration of remission, although in 75% of the cases at least one major rearranged IgH, TCR $\gamma$  or TCR $\delta$  allele remained the same at relapse. Clonal evolution and oligoclonality in IgH genes are caused by disruption in the V-N-D portion, whereas D-N-J sequences are left unmodified. Davis et al (1991) analyzed the IgH gene by PCR at diagnosis and relapse in 12 cases of ALL and detected clonal evolution in 4 cases attributed to VH gene replacement and in 3 cases due to new rearrangements with loss original alteration. Stewart et al, identified changes in the pattern of PCR amplification between diagnosis and relapse in 12 of 39 patients with B-lineage ALL studied for

IgH gene rearrangements. In 9 of 12 cases, there were observed subclones or rearrangements of partial or complete configurations determined at diagnosis. However, major pitfalls of this application are the occurrence of multiple rearrangements at diagnosis (oligoclonality) and modified patterns that appear at relapse (clonal evolution), which may lead to false negative results of MRD-PCR technique (Van Dongen et al, 1991).

PCR based clonality testing in lymphoproliferations combined with immunohistology and with results from flow-cytometric immunophenotyping offer an integration of all available data to reach the most reliable diagnosis.

## CONCLUSIONS

Accurate determination of MRD had a profound impact in the clinical management practices of patients with hematologic malignancies. Prospective studies in large series of patients have demonstrated a strong correlation between MRD levels during clinical remission and treatment outcome. Therefore, MRD assays can be used to assess early response to treatment and predict relapse. To conclude, there is a need to integrate the molecular data with data from immunohistology and also flow-cytometric immunophenotyping for an appropriate interpretation and treatment effectiveness.

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