Introduction

One of the most obvious applications of minimal residual disease (MRD) testing in acute leukemia is its use in measuring early treatment response and identifying patients who achieve morphologic remission but still harbor considerable levels of disease ***(Campana, 2009).***

The term "MRD" was created to describe disease that was detected only by laboratory techniques more sensitive than morphology, such as flow cytometry (immunologic MRD) or polymerase chain reaction (PCR) (molecular MRD). This residual disease was believed to be minimal, given that it was found in the absence of clinical signs or symptoms ***(Paietta and Papenhausen, 2001).***

Obviously, the ultimate goal of MRD assays is to guide therapeutic decisions by recognizing patients who responded well to therapy and, thus, should be spared further therapy, and distinguishing them from patients in whom therapy must be continued or intensified to minimize the likelihood of clinical relapse ***(Malec et al., 2001).***

The PCR amplification of genes is a reliable and accurate method for monitoring MRD and can be used in the majority of cases of childhood and adult acute leukemia ***(Van der Velden et al., 2007).*** Moreover, a positive MRD result with this technique may alert to the presence of pre-leukemic

or “leukemia-initiating” cells, which might be missed by other methods ***(Hong et al., 2008).***

Stanniocalcin-1 (STC-1) is a secreted glycoprotein hormone; its high expression has been associated with several cancers ***(Liu et al., 2010).*** The STC-1 expression is observed in malignant tissues such as adrenal medulla, hepatocellular carcinoma and breast cancer, suggesting some roles in malignantly-transformed cells ***(Wascher et al., 2003).***

The PCR analysis showed high levels of STC-1 mRNA in the blood of patients with cancer as compared with those in the blood of volunteers without cancer ***(Koide and Sasaki, 2006).***

The STC-1 mRNA is detectable in phytohemagglutinin-stimulated T-cells and in most human leukemic cell-lines, suggesting a role of STC-1 for cell proliferation. This finding prompts the usefulness of STC-1 for monitoring acute leukemia ***(Tohmiya et al., 2004).***

Aim of the Work

The aim of this study is to:

a) Evaluate the Stanniocalcin-1 gene expression in patients with acute leukemia.

b) Its ability to monitor MRD in such patients.

c) Compare its value in detection of MRD against the routinely used methods.

Chapter (1)

MINIMAL RESIDUAL DISEASE

Definition:

Minimal residual disease (MRD) is defined as any measurable disease or leukemia detectable above a certain threshold (defined by the methodology applied), predicts failure to maintain a morphologic complete remission (CR), and affects survival negatively ***(Paietta, 2012)***. It is worth noting that MRD at a level of 0.0001% can still equate to 1,000,000 leukemia cells within the body. Detecting and measuring MRD is important because it can help to guide treatment decisions. The MRD has no defined numeric value, but in common practice the term describes a level of disease beyond the resolution of microscopic techniques, the most sensitive of these being fluorescent in situ hybridisation (FISH) which can detect one leukemic cell in a background of 500 normal cells (0.2%) ***(Mason and Griffiths, 2011)***.

The assessment of MRD has gained a prominent position in many acute leukemia treatment studies, as a tool for tailoring therapy. Measurement of submicroscopic (minimal) levels of MRD can be used to monitor treatment response much more precisely than morphological screening of bone marrow (BM) slides. The detection of MRD is particularly useful not only for evaluation of early treatment response, but also to monitor disease before and after stem cell transplantation (SCT), for

early assessment of an impending relapse, and in the setting of salvage treatment. Currently, three highly specific and sensitive methodologies for MRD detection are available, namely, real-time quantitative polymerase chain reaction (RQ-PCR) of fusion gene transcripts or breakpoints, RQ-PCR–based detection of clonal immunoglobulin and T-cell receptor (TCR) gene rearrangements, and multi-parameter flow cytometric (FCM) immunophenotyping (IPT) ***(Brüggemann et al., 2012).***

Prevalence of MRD:

Acute lymphoblastic leukemia (ALL) is the commonest malignancy in children (aged 1–18 years), accounting for almost 30% of all cancers in this age group. Notable improvements in the treatment of childhood ALL have been made in the past four decades **(Conter et al., 2010),** compared with the late 1960s, when 30% of cases were cured. Nowadays about 80% of patients remain in their first remission even after 10 years. The remaining 20% of children have recurrences, and the cure rate falls to 25–40%. Conversely, it is also discussable that a proportion of the cured patients are currently over-treated and would benefit from a reduction in treatment for the sake of lower toxicity and fewer long-term sequelae.

In addition to determining patient-specific risk factors (such as sex, age) and ALL-specific risk factors (such as leukocyte count, IPT, chromosomal aberrations) when making the diagnosis, in vivo confirmation of responsiveness to chemotherapy has been found to be of particular relevance in

predicting the likelihood of survival and risk of recurrence ***(Bartram et al., 2012).***

The achievement of complete hematologic remission (CHR) is a prerequisite for cure in acute myeloid leukemia (AML). The conventional definition of CR, based on the morphologic recognition of ≤ 5% of leukemic blasts in the BM, does not provide sufficient insight into the quality of the response. Despite CR rates of 50%-80% (depending on age), the majority of patients with AML relapses within 3-5 years from diagnosis. Therefore, there is great need of more sensitive prognostic factors that can predict relapse. The AML is lagging behind ALL with respect to the implementation of MRD criteria for guidance during therapy. The AML is particularly disadvantaged compared with ALL in that approximately half of AML patients lack a molecular target suitable for MRD monitoring. The detection of altered Ag expression by leukemic myeloblasts is a valid alternative to DNA- or RNA-based MRD assays. Although associated with presenting prognostic factors (e.g., cytogenetics and genotype), MRD represents the collective end-result of all of the cellular mechanisms that determine a patient's response to a given therapy. Therefore, MRD has two potential roles in AML treatment: **first** as a post-therapy prognosticator used to assign patients to optimal post-induction/consolidation therapy, and **second** as an early surrogate end-point for the evaluation of therapy efficacy ***(Paietta, 2012).***

The clinical significance of MRD:

As a post-therapy prognosticator, MRD status has variable prognostic power depending on the time of assessment ***(Paietta, 2012).*** Although rapid tumor clearance after therapy initiation is of critical value ***(Rubnitz et al., 2010),*** some investigators have found that post-consolidation MRD levels carry superior prognostic power.

Therefore, there are distinct approaches to the use of the information provided by MRD. One approach relies on MRD detection early on, after induction therapy, to refine risk stratification, which otherwise relies mostly on pre-treatment parameters. Early evaluation of disease response may also be of particular interest in the context of novel, investigational agents. Risk-directed therapy not only aims at improving outcome, but also at avoiding excessive toxicities by not exposing patients unnecessarily to additional treatment. The other approach uses serial monitoring of MRD during hematologic remission in an effort to prevent the development of clinical relapse by reacting to increasing MRD levels with pre-emptive therapy ***(Buccisano et al., 2012).***

It is still a matter of debate what is the best method to use to measure MRD? The various methods are compared based on their sensitivity, specificity, and applicability to the largest number of patients. Although MRD is quantified as a continuous variable reflected by the percentage of blasts among all normal nucleated cells, the absolute levels of leukemia

transcripts or mutated genes or their log change with treatment, thresholds are regularly defined for clinical decision-making. Therefore, patients are classified in a dichotomous manner as high/positive MRD (above a given threshold) or low/negative MRD (below a given threshold). Whereas this may not always be biologically sound, it facilitates the use of MRD results in guiding therapeutic decisions. The chosen threshold determines both sensitivity and specificity of a MRD assay ***(Steinbach and Debatin, 2008).***

Sensitivity reflects the assay's ability to classify patients as high MRD (i.e., when the number of leukemic blasts in a tissue is higher than the set threshold); specificity reflects the assay's accuracy in defining patients with low MRD (i.e., when the number of leukemic cells in a tissue is lower than the set threshold) ***(Rossi et al., 2012).***

The MRD thresholds with prognostic relevance are highly dependent on methodology (molecular or IPTic), MRD target, and trial design and, unless standardized, are difficult to compare across reports. Using multicolor antibody (Ab) combinations, the limit of detection with multi-parameter FCM can reach 10^{-4} (0.01%) depending upon the leukemia associated immunophenotype (LAP) used and sample quality, level comparable to that achieved by reverse transcription (RT-PCR) methodology for measuring molecular targets.

Sequential monitoring of MRD using sensitive and specific techniques, such as PCR and FCM analysis, with a

detection power of one blast cell in 10^3 to 10^6 normal cells, has substantially refined the assessment of early response to treatment. These methods, however, are expensive and complex and require considerable technological availability, thus being inaccessible to most treatment centers, especially in developing countries. Simplified methodologies for the assessment of the early response, including the evaluation of MRD, may provide good predictive criteria of an unfavorable course in children with ALL and could be used to identify patients at a high risk of relapse ***(Scrideli et al., 2009).***

Although RQ-PCR for leukemia fusion transcripts is a very sensitive way to detect MRD, the clinical significance of a positive result (specificity) can vary with the time of assessment during the clinical course ***(Rossi et al., 2012).***

Moreover, increased sensitivity for MRD monitoring is particularly important post-allogeneic hematopoietic stem cell transplantation (HSCT), when presence or rise in MRD is likely to be predictive of relapse **(Verneris and Burke, 2010).**

Methods for Detection of MRD

The standard method to gauge the therapeutic response is cyto-morphology by means of microscopy. Its disadvantage is its low sensitivity, which, in the best case scenario, allows detection of one leukemia cell among 100 normal cells. According to this diagnostic criterion, 95–98% of patients achieve CR within four weeks of starting therapy. This means

that in substantial proportions, intensive poly-chemotherapy was administered after the leukemia had become undetectable. Two decades ago, a diagnostic break-through was reached when polymerase chain reaction (PCR) was introduced to identify minute amounts of malignant cells (MRD) ***(Bartram et al., 2012).***

Three methods for identifying minute amounts of malignant cells MRD allow the identification of one leukemia cell among 10,000 to one million normal cells (10^{-4} to 10^{-6}) and are, thus, 100–10,000 times more sensitive than conventional cyto-morphological techniques. These methods differ with regard to their applicability (frequency of relevant target structures) in terms of their sensitivity and their expense of time and money ***(Brüggemann et al., 2010):***

- **IPT using FCM and multiple markers** to identify leukemia-specific immunophenotypes.
- **PCR analysis of fusion products of leukemia specific chromosomal translocations** (e.g., BCR-ABL recombination of the Philadelphia (Ph) translocation- t(9;22), and PCR analysis of clone-specific chain joining regions of immunoglobulin (Ig) and TCR rearrangements.

In the meantime, a third method has become most widespread in Europe because of its excellent sensitivity. This method is based on the insight that ALL always starts from individual lymphocyte precursors, and all leukemias are, therefore, characterized by an individual, clone-specific pattern

on Ig rearrangements or TCR rearrangements. The molecular basis is a **DNA rearrangement** in which different segments of the Ig genes or TCR genes are joined individually. In the context of these re-combination processes, fewer or more nucleotides from the original DNA sequence are lost at the joining regions, and free nucleotides can be inserted into this region. The result is a unique DNA sequence for a particular lymphocyte, which can be identified on the basis of PCR analysis by using allele-specific DNA probes- a method that many working groups have been developing since the late 1980s in order to measure MDR in patients with ALL. Since ALL can display subpopulations of an initial leukemia cell clone (oligo-clonality) at the time of diagnosis, and may undergo further Ig- and TCR-rearrangements during the disease course, which may then result in the loss of the DNA sequences used as probes, it is recommended for the PCR analysis to use two or more target sequences in order to avoid false-negative results ***(Bartram et al., 2012)*** (table 1).

Table (1): Characteristics of the Techniques Currently Employed for MRD Detection in ALL

Characteristics	PCR Analysis of Ig &TCR Gene Rearrangements	PCR Analysis of BCR-ABL Transcripts	Multiparameter FCM
Sensitivity	RQ-PCR: 10^{-4}–10^{-5}	10^{-4}–10^{-6}	3- to 4-color: 10^{-3}–10^{-4} 6- to 9-color: 10^{-4}–10^{-5} Depends also on cell-input
Quant. range	RQ-PCR: 10^{-2}–10^{-4}	Not yet defined	Not yet defined
Applicability	Precursor-B-ALL: 90%–95% T-ALL: 90–95%	Ph^+ ALL (5%–8% of children with precursor -B-ALL, 30%–35% of adults with precursor-B-ALL)	Precursor-B-ALL: 80%–95% T-ALL: 90%–95% Depends also on number of colors
Advantages	● High sensitivity ● High degree of standardization ● Well-established stratification tool in clinical protocols ● Evidence based treatment decisions ● Applicable for almost all ALL pts. ● Stability of DNA (multicenter setting)	● High sensitivity ● Stability of target during course of treatment ● Fast ● Relatively cheap	● Applicable for almost all ALL patients ● Rapid ● Quantitative ● Additional information on benign cells ● Additional information on malignant cells ● Growing standardization throughout Europe
Disadvantages	● Time-consuming marker character-ization ● Potential instabil-ity of targets (clonal evolution phenomena) ● Extensive knowledge & experience needed ● Relatively expensive	● Applicable only in Ph^+ patients ● Instability of RNA ● Possible differences in expression levels ● Standardization necessary ● Risk of false positivity due to contamination	●IPTic shifts. ● Expanded precursor-B-cell compartment during regeneration. ● Low cellularity during/ after induction. ● Relatively expensive (depends on number of markers/colors & FCM utilizations). ● Limited sensitivity/ applicability using 3- to 4-color FCM. ● 6-color FCM: needs Extensive knowledge & experience for sensitive & standardized analysis.

Quant. Range: quantitative range

(Brüggemann et al., 2012).

A) FCM:

- Targets for FCMic studies

Leukemic cells express IPTic features that can be used to distinguish them from normal hematopoietic cells, especially from physiologic lymphoid precursors present in the BM. These LAPs can be grouped into different categories:

First, the appearance of immature phenotypes outside their normal tissues, which can be exploited in particular in T-ALL: early T-cell development takes place in the thymus and therefore detection of (TdT^+ and/or $CD34^+$) T-precursors in the BM or blood signifies the presence of T-ALL cells.

Second, the LAPs can be represented by markers that are also expressed in benign hematogones but found in abnormal combination with qualitative (eg, expression of CD66c, CD13, CD33, NG-2, CD21) or quantitative (eg, under-expression of CD10, CD38, CD45, and over-expresssion of CD58) differences in expression patterns compared to benign hematogones ***(Brüggemann et al., 2012).*** This approach is most widely used to monitor MRD by FCM, and it is currently the only one that sensitively tracks B-lineage leukemic cells.

Third, fusion proteins resulting from chromosomal translocations are attractive targets, as they are leukemia specific. However, despite the efforts to produce Abs against these proteins, there are currently no such Abs available for routine diagnostics. A FCM immune-bead assay for detection of BCR-ABL proteins in cell lysates reaches sensitivities of about 1%, making this approach currently mainly suitable for diagnosis and

classification of leukemias, but limiting its applicability for sensitive MRD monitoring ***(Weerkamp et al., 2009).***

Using 4-color FCM, LAP can be identified in about 90% of B-precursor and more than 95% of all T-ALL patients, reaching a detection limit of 10^{-3} to 10^{-4}. Sensitivities are currently about one log less than molecular methods, because MRD positivity cannot be determined at the single-cell level by FCM. To be unambiguous it needs to demonstrate a certain amount of cells with similar (leukemia-associated) characteristics ("cluster") ***(Brüggemann et al., 2012)***.

The analysis of IPTic aberrancies with FCM, as a measure of MRD, presents with its own challenges. In AML, unlike ALL, multiple LAPs are detected regularly on subsets of blasts in individual patients at diagnosis. Comparisons of paired presentation/relapse samples often show selective LAP changes. Such IPTic shifts will not affect the utility of FCM for MRD detection, provided that as many independent LAPs as possible are monitored per patient ***(Ossenkoppele et al., 2011).*** This approach also reduces the likelihood of false-positive MRD results due to the potential presence of LAP Ag combinations at low frequencies in normal BM, BM after chemotherapy, or after growth factor administration ***(Jorgensen and Chen, 2011).***

Although both specificity and sensitivity of detection of many LAPs is limited by background, claims that MRD by FCM will consistently produce low-level positivity in normal or treated BM are unfounded ***(Steinbach and Debatin, 2008).***

Six (or more)–color technology and comprehensive Ab combinations, including Abs to leukemic stem cells (LSCs) and multiple lymphoid Antigens (Ag), allow for the construction of LAPs with the highest specificity ***(Buccisano et al., 2012).*** Granted, depending on the expertise of the FCM operator, Ag expression patterns may be misinterpreted. In this regard, standardized protocols and automated data file analyses may be particularly useful for MRD detection in AML ***(Schuurhuis and Ossenkoppele, 2010).***

In experienced laboratories, a positive MRD result by FCM rests on the identification of only 20 clustered abnormal events. Because leukemic cells are quantified in relation to other cells in the specimen, the smallest abnormal cell cluster that can be reliably called MRD depends on the total number of cells analyzed. With 200,000 cells acquired, a 20-cell cluster represents a sensitivity of one in 10^4 (0.01%). The lower the number of abnormal cells present, the higher the number of cells required to be analyzed. As a result, if the sample quantity is limited, the sensitivity of the MRD assay will be lowered ***(Freeman et al., 2008).*** This stresses the importance of sample quality for accurate MRD evaluation.

Antibodies suitable for MRD detection should (1) distinguish leukemic blasts from normal myeloid precursors; (2) detect lineage-foreign markers (e.g., lymphoid-affiliated Ags, such as CD7 and CD19); (3) detect altered density of myeloid or lineage-uncommitted Ags compared with normal myeloid precursors (e.g., CD33 and CD11a), or (4) detect asynchronous expression of Ags (e.g., CD123 and CD34) (figure 1).