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Review article

Methods of minimal residual disease (MRD) detection in childhood haematological malignancies

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Abstract. The appropriate management of haematological disorders must rely on a precise and long-term monitoring of the patient's response to chemotherapy and radiotherapy. Clinical data are not sufficient and that is why in the last decade it became the most important to improve the knowledge of haematological diseases on the basis of molecular techniques and molecular markers. The presence of residual malignant cells among normal cells is termed minimal residual disease (MRD). Nowadays a great progress has been made in the treatment of malignant diseases and in the development of reliable molecular techniques, which are characterised by high sensitivity (10^{-3}– 10^{-6}) and ability to distinguish between normal and malignant cells at diagnosis and during follow-up. Especially, MRD data based on quantitative analysis (RQ-PCR, RT-RQ-PCR) appear to be crucial for appropriate evaluation of treatment response in many haematological malignancies. Implementation of standardized approaches for MRD assessment into routine molecular diagnostics available in all oncohaematological centres should be regarded nowadays a crucial point in further MRD study development.

Keywords: Ig/TCR rearrangements, malignancy, minimal residual disease, prognosis, RQ-PCR.

Introduction

Over the past 30 years, remarkable progress has been made in the treatment and understanding of the biology of haematological malignancies, especially of childhood lymphoid and myeloid leukaemias. With the contemporary improved risk assessment, chemotherapy, haematopoietic stem cell transplantation (HSCT) and supportive care, about 80% of children with newly diagnosed acute lymphoblastic leukaemia (ALL), 60% of patients with CML (chronic myeloid leukaemia), 50% with AML (acute myeloid leukaemia), and 70% of patients with Hodgkin's disease can be cured currently (Faderl et al. 1999). Despite the great advances in the cure rate for ALL, in about 20% of affected children the disease recurs, which is the main reason of therapeutic failures.

Failure to achieve sustained remission in children with ALL and other malignancies is in most cases caused by the survival of chemotherapy-resistant neoplastic cells, which are responsible for the relapse. The presence of residual malignant cells among normal cells is termed minimal residual disease (MRD) (Faderl et al. 1999; Szczepański et al. 2001; Raanani and Ben-Bassat 2004). These malignant cells are often below the limits of detection of cytomorphological techniques. Hence, detection of low levels of malignant cells with molecular techniques became the crucial tool of modern haematological diagnostics (Kaeda et al. 2002; Hillmen, 2006).

After the induction phase, about 80–90% of patients experience complete remission, as confirmed by microscopic examination, where blast cells make up less than 5% of bone marrow smear, which is equivalent to 10^{10} of neoplastic cells. However, it should be noted that these figures represent, in fact, borderline sensitivity of detecting a neoplastic process by microscopic cytological

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examination. As regards cytological evaluation, the key problem is uncertainty whether the remission that has been attained is complete. It seems that a more reliable answer can be obtained with the development of more precise criteria of remission quality evaluation during each phase of the treatment and after its completion. Therefore, MRD level detection enables evaluation of treatment effectiveness in haematological disorders and also represents a powerful prognostic factor helping to predict the clinical outcome (Campana et al. 1999; Coustan-Smith et al. 2002; Willemse et al. 2002; Malec et al. 2004).

Because of the major role of MRD in the pathogenesis of relapse, several new methods of evaluation, characterised by increased sensitivity of MRD detection, have recently been developed. Some of these techniques offer the sensitivity of MRD detection of 1 neoplastic cell per $10^4 – 10^6$ normal cells, which makes them $10^2 – 10^4$ times more sensitive than cytomorphological techniques currently used to confirm remission.

The main goal therefore is to monitor MRD by using the most sensitive techniques, which enable reliable assessment of patients' response to treatment and to identify early symptoms of relapse (Zwick et al. 2006).

Clinical aspects of MRD detection

Detection of MRD has applicability in clinical practice due to its important prognostic value in a number of haematological diseases, e.g. ALL, AML, CML (van der Velden et al. 2003; Steinbach et al. 2006). Many studies have shown that MRD levels significantly correlate with the patient's clinical outcome (Dibenedetto et al. 1997; Bader 2002; Willemse et al. 2002). In ALL, for example, quantitation of MRD levels at stated time-points during therapy enables classification of patients into risk groups, i.e. identification of patients at high (MRD levels $\geq 10^{-3}$), intermediate, and low risk (no MRD detection) of relapse (Dawidowska et al. 2006; van der Velden et al. 2006). Introduction of MRD-based protocols may also improve the therapy of AML (Goulden 2006). Moreover, detection of MRD identifies differences in treatment response between patients with T-ALL and precursor-B-ALL (Szczepański et. al 2001). The most important seems also individualization of treatment between T-ALL and pre B-ALL, on the basis of results of MRD analysis (Willemse et al. 2002).

On the other hand, the most sensitive techniques of MRD detection can better confirm the state of remission and are useful for appropriate selection of a cure strategy for each particular patient, which can improve long-term survival (Schuler et at. 2006). Others show that MRD results strongly help to predict relapse in patients suffering from acute promyelocytic leukaemia (APL) (Huang et al. 1993; Goulden 2006) or AML (Szczepański et al. 2001). Sometimes knowledge about MRD levels at particular points of medical management enables early or more aggressive treatment intervention (Hillmen 2006).

In patients after allogeneic bone marrow transplantation, a high level of MRD prior to transplantation can usually indicate the risk of disease relapse. Several studies confirm a strong correlation between pre-transplant MRD level and post-transplant clinical outcome, especially in ALL cases (Knechtli et al.1998; Bader et al. 2002; Uzunel et al. 2003). Sometimes, MRD results can also help physicians in making additional therapeutic decisions or in better monitoring of disease development, especially in patients after non-myeloablative allogeneic transplantation (Galimberti et al. 2005). Post-transplant MRD-based monitoring of treatment response was also reported for CML patients (Kaeda et al. 2002).

Techniques for MRD assessment

Since MRD means the presence of leukaemic cells among normal cells, techniques used for MRD detection rely on finding leukaemia-specific markers, which distinguish between leukaemic blasts and normal cells. There are various techniques applicable for MRD detection, which differ in specificity of markers used as well as detection levels. Some of them are limited by their low sensitivity: morphology of the cells, clonogenic assays, and conventional cytogenetics. Classical cytogenetics has been successfully replaced by other techniques, like fluorescent *in situ* hybridization (FISH) or RT-PCR (Kaeda et al. 2002). Nowadays, the modern, highly sensitive methods used in the detection of MRD are: quantitative polymerase chain reaction (RQ-PCR), quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) and flow cytometry (Munoz et al. 2000; Szczepański 2001).

Cytomorphology is still a standard technique for identification of complete remission but the detection limit is $10^{-1} – 10^{-2}$. This routine technique is based on assessment of morphology of bone marrow cells with the use of a light microscope (Toren et al. 1996). The same sensitivity represents a cytogenetic analysis based on an anal-

ysis of chromosomal morphology. This method strongly depends on a number of metaphases obtained from a blood or marrow sample. The most common cytogenetic abnormalities in ALL and AML are presented in Table 1. Sometimes detection of chromosomal abnormality is not possible, due to technical problems with culturing the cells to metaphase or mostly because of a low quality of the material obtained from the hospital.

FISH is a method to detect the absence or presence of additional chromosomes and also to detect or confirm gene or chromosome changes (detection of fusion genes: *BCR-ABL, TEL-AML, MLL-AF4, PML-RAR, AML1/ETO*) (Ładoń et al. 2001; Raanani and Ben-Bassat 2004). FISH relies on hybridization of specific fluorescently labelled probes to the metaphase chromosomes or interphase nuclei. In a case of translocation a specific molecular probe applied spans the site of breakage and is complementary to the known sequence. The fluorescently labelled probe hybridizes with the sample DNA at the target site and then signals can be seen under a fluorescent microscope. For example, FISH is routinely used for detection of the BCR-ABL fusion product as a result of t(9;22). Detection limits of fluorescence in situ hybridization are 10^{-2}– 10^{-3} (Liang et al. 1997).

Flow cytometry is used for MRD detection to quantitate malignant cells present in the patient's blood or bone marrow sample. Flow-cytometric detection of MRD is based on the identification of immunophenotypic combinations expressed only in malignant cells but not on normal bone marrow or peripheral blood cells (Campana and Coustan-Smith 1999; Faderl et al. 1999; Coustan-Smith et al. 2002). Cells are incubated with monoclonal antibodies, which recognize only antigens expressed on the surface of the malignant cells. The antibodies are labelled with various fluorochromes, crucial for detection, and then counted by a flow cytometer, a laser-based instrument. This method can detect 1 leukaemic cell per about 10^4 normal cells (Campana and Coustan-Smith 1999; Liang et al. 1999; Coustan-Smith et al. 2002).

Table 1. The most common cytogenetic abnormalities in acute lymphoblastic and acute myeloid leukaemias

Acute lymphoblastic leukaemia (ALL)		Acute myeloid leukaemia (AML)
B-line lymphocytes	T-line lymphocytes	
t(9;22)(q34;q11)	t(1;7)(p32;q34)	t(9;22)(q34;q11)
t(4;11)(q21;q23)	t(1;14)(p32;q11)	t(8;21)(q22;q22)
t(1;19)(q23;p13)		t(15;17)(q22;q11-21)
		t(16,16)(p13;q22)
		t(3;21)(q26;q22)

PCR-based methods of MRD detection

PCR-based techniques for the monitoring of MRD rely on detection of 2 kinds of targets: (1) breakpoint regions of leukaemia-specific chromosomal aberrations, and (2) immunoglobulin or T-cell receptor gene rearrangements (Gemano et al. 2003; Li et al. 2003). The higher sensitivity of PCR enables detection of 1 leukaemic cell among 10^5– 10^6 normal cells (Willemse et al. 2002).

During B-cell and T-cell differentiation, the germline gene segments (V-variable, D-diversity, J-joining) of the immunoglobulins and T-cell receptors, rearrange. Each mature lymphocyte obtains a specific combination of V-(D)-J segments. The process of recombination is imprecise. During joining of the gene segments, random nucleotides are inserted or deleted. The whole repertoire of antigen-specific receptors depends on combinatorial and junctional diversity. Combinatorial diversity is a simple consequence of possible combinations of all available gene segments: V, D and J. Junctional diversity relies on the presence of spontaneous insertions between the joined segments or deletions of a single or a few nucleotides from the germline sequence. As a result of random nucleotide insertions and deletions at the junction sites of the V, D, J gene segments, the sequence at junctional regions is fingerprint-like and can be used as a specific marker of each lymphocyte clone, and thus a specific molecular marker of residual leukaemic cells. Since leukaemias are regarded as clonal cell proliferations, these rearrangements are expected to be identical in all cells derived from a particular leukaemia. The rearrangements of leukaemia blasts detected at diagnosis are target sequences for the monitoring of MRD in follow-up samples. The PCR reaction is per-

formed with the use of a set of primers designed for particular V, D, J segments. PCR products are analysed in heteroduplex analysis for discrimination between monoclonal (leukaemia-derived) and polyclonal (normal lymphocytes-derived) PCR products. After heteroduplex analysis, clonal products are sequenced to identify the junctional sequence of each specific clone, for which an oligonucleotide probe or a primer is designed. MRD monitoring, based on quantification of MRD levels, is then performed with the use of real-time quantitative polymerase chain reaction (RQ-PCR) and designed primers/probes (Szczepański et al. 2001; Li et al. 2002; Gemano et al. 2003; van der Velden et al. 2003). RQ-PCR enabling the monitoring of the progress of PCR in real time completely revolutionized the methodology of MRD analysis. Real-time quantitative polymerase chain reaction is based on detection of a fluorescent signal produced proportionally to amplification of a PCR product. The detection systems differ in their chemistry. In TaqMan technology the probe, localized after hybridization between the 'traditional' reverse and forward primer, is an oligonucleotide with a reporter fluorescent dye and a quencher dye attached (usually a FAM- TAMRA fluorochromes). As long as the probe is intact, no fluorescence is emitted. During amplification, the probe annealed to the target sequence is cleaved by the 5'-nuclease activity of Taq DNA polymerase, which results in emission of fluorescence, whose intensity is proportional to the amount of the PCR product amplified. This is used for detection of amplification of the target-specific product and for quantitative analysis. While the probe is hybridized to the template, the quencher reduces the fluorescence emitted by the reporter dye. Cleavage of the probe separates the reporter and the quencher dyes and thus increases the fluorescence of the reporter. In each PCR cycle the intensity of fluorescence is increasing proportionally to the amount of amplicon produced. Analysis of clone-specific immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements as target sequences for MRD detection can be also performed with the LightCycler and SYBR Green strategy, alternative to the TaqMan system (Li et al. 2002). The technique combines rapid thermocycling with online fluorescence detection of PCR products. In this technique, leukaemia-specific PCR products are measured at each cycle by staining the PCR products with the SYBR Green dye, which intercalates between double-stranded DNA (Nakao et al. 2000). Fluorescence emitted by SYBR Green is measured by the system.

Comparison between RQ-PCR analysis of immunoglobulin and TCR gene rearrangements and multicolour flow cytometric immunophenotyping shows that MRD levels may differ and these techniques are not exchangeable (Malec et al. 2004). However, some authors show that although RQ-PCR is more sensitive than flow cytometry, both techniques provide concordant prognostic results (Kerst et al. 2005; Robillard et al. 2005).

MRD analysis based on detection of clonality can be also performed with the use of ligase chain reaction (LCR). This is a DNA amplification technique based upon the ligation of oligonucleotide probes. The probes are designed to match exactly 2 adjacent sequences of a specific target DNA. The sensitivity of LCR is 1 malignant cell per 500 000 cells (Jilani et al. 2006).

Clonal stability of rearrangements appears the most important. Important information about Ig/TCR target stability is provided by GeneScan analysis after sequencing analysis of junctions at diagnosis and relapse. It is an excellent technique for determining the size of PCR products and distinguishing between clonal and polyclonal targets. It becomes crucial when relapse is recognized. Moreover, it enables to recognize if the Ig/TCR clonality profile results from a primary or a secondary malignancy (Szczepański et al. 2002; Gemano et al. 2003). Some authors conclude that comparison of Ig and TCR gene rearrangements in precursor-B-ALL children at diagnosis and during recognition of disease relapse did not show any significant difference in their stability. Hence there is no preferential clone-specific target for MRD detection (Gemano et al. 2003). However, others regard Ig/TCR gene rearrangements as excellent targets for the monitoring of MRD in ALL paediatric patients but also highlight that they might be unstable during disease treatment (Li et al. 2003). Basing on this, they propose strategy for selection of stable PCR targets for MRD monitoring, with a special successful detection of relapse in almost 95% of patients (Szczepański et al. 2002). Always 2 or even more targets should be used per patient in order to avoid false-negative results (Szczepański et al. 2002; Li et al. 2003). Monoclonal targets represent a high stability and should be chosen as the first option. The second choice determines oligoclonal IGH targets (Szczepański et al. 2002). In precursor-B-ALL the following order is preferred: *IGK*, *IGH*, *TCRB*, *TCRD* or *TCRD/A*, and then *TCRG*, while in

T-ALL: *SIL-TAL*, *TCRD*, *TCRB*, *TCRG*. Regardless of the preferred order of gene target selection, information on junction region sequence should be considered.

Aberrant genes and aberrantly expressed genes can also be used as specific targets for MRD detection, e.g. the *WT1* gene, which encodes a zinc finger peptide and is a tumour suppressor gene (van der Velden et al. 2003). The *WT1* gene is overexpressed in about 70–90% of all acute leukaemias (Boublikova et al. 2006). Hence, the expression level of the *WT1* gene monitored with the use of the RQ-PCR technique is an excellent tool for detection of MRD in acute leukaemias and also in other malignancies (Lapillonne et al. 2006; Rodrigues et al. 2006; Tamura et al. 2006). However, other authors indicate that in the case of childhood ALL, *WT1* is not a useful marker for MRD detection (Gaiger et al. 1999; Boublikova et al. 2006), but can be regarded as a potential risk factor (Boublikova et al. 2006). However, the *WT1* gene is being used as a prognostic factor, especially in fusion-transcript-negative leukaemias (Kletzel et al. 2002; Lapillonne et al. 2006; Steinbach et al. 2006).

MRD can be also detected by reverse transcriptase polymerase chain reaction (RT-PCR), e.g. nested RT-PCR. Nested RT-PCR consists of reverse transcriptase reaction and sequential double PCR. It is a modification of the 'traditional' PCR technique and is used to improve its sensitivity. Two pairs of PCR primers are used for amplification of a single locus. The first pair is designed on target mRNA and then the second pair of primers (named nested primers) bind within the first PCR product and produce a second PCR product, shorter than the first one. This modification raises sensitivity up to 10^{-5}– 10^{-6} (Kaeda et al. 2002). Different fusion transcripts specific for particular leukaemias (e.g. *BCR-ABL, CBFbeta/MYH11* or *AML1-ETO*, Raanani and Ben-Bassat 2004) can be detected by using this technology. Fusion transcripts detected after recognition of malignancy are then used as markers for the monitoring of MRD in follow-up samples.

For analysis of the kinetics of MRD, semiquantitative competitive RT-PCR has been developed. This technique is based on the quantification of MRD to the level of expression of an endogenous control gene. Competitive PCR involves the co-amplification of an internal control (named competitor) at various but known concentrations, together with the target sequence (Syvanen 1999). The assay described is useful for estimation of the number of fusion transcripts in the particular leukaemia type (e.g. the number of *BCR-ABL* transcripts in CML patients) to monitor MRD after transplantation or during medical treatment (Cross et al. 1993). When haematological relapse occurs, the higher level of fusion gene transcripts is observed.

Nowadays, a standard technique used in both children and adults for the monitoring of fusion transcripts, e.g. *BCR-ABL* in CML, *TEL-AML1* in ALL, *PML-RARalpha* in APL or *AML1-ETO* in AML, is quantitative real-time reverse transcriptase polymerase chain reaction (QRT-PCR) (Viehmann et al. 2003; Hardling et al. 2004; Stock et al. 2006; Mokany et al. 2006; Goulden 2006). This technology provides the most accurate assessment of MRD in follow-up samples with a high sensitivity of 10^{-5}– 10^{-6} (Hardling et al. 2004; Stock et al. 2006; Mokany et al. 2006). The *TEL-AML1* fusion gene caused by t(12;21)(p13;q22) is present in 25% of children with precursor-B-ALL. In these patients quantitative RT-PCR analysis of the *TEL-AML1* fusion transcripts can be sufficient for MRD detection and can easily replace the analysis of quantitative Ig/TCR rearrangements (De Haas et al. 2002).

Conclusions

From the biological and medical point of view, the monitoring of MRD creates novel problems, such as: which MRD strategy is the most reliable (sensitivity is the most important factor), or which strategy shows the greatest predictive value. Reliable techniques must distinguish patients who are destined to relapse and who could benefit from additional therapy. On the other hand, in patients from a low-risk group, less aggressive treatment intervention should be considered. There is definitely an urgent need for ongoing studies of MRD to improve understanding of clinical applicability of various technologies of MRD detection. Nowadays, dissemination of a routine molecular diagnostics of MRD in most of haematological centres becomes an absolute necessity.

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