Quantitative analysis of minimal residual disease by PCR in childhood acute lymphoid leukemia

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Chapter 1

GENERAL INTRODUCTION

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Improvement of therapy for children with acute lymphoid leukemia (ALL) has resulted in survival rates of over 80%; however, 20-30% of children ultimately still suffer from a relapse. In standard patient care, risk assessment is mostly performed at diagnosis. In order to increase survival with treatment intensification in high-risk patients, individualization of treatment is needed, preferably in combination with less therapy in low-risk patients to reduce side effects. New prognostic markers added to the current prognostic factors, i.e. age at presentation, white blood cell count, immunophenotype, DNA-ploidy and chromosomal abnormalities may allow better stratification of patients. Several reports demonstrated that levels of minimal residual disease (MRD) after induction therapy and early during treatment are very important prognostic factors in relation to ultimate outcome in childhood ALL. As a result the level of residual disease after induction of remission as determined by quantitative PCR or flow cytometry, might be used to discriminate between patients at high, intermediate or low risk for relapse. This MRD-based risk classification can be used to tailor treatment.

Over the last few years many people have studied the detection of MRD. Four main questions are ever recurring. Is it possible to detect and to quantify MRD in children with ALL and can this add to therapy-stratification and outcome of patients? What is the preferable technical approach? Which time-point(s) is (are) the most informative? And finally, what is the most simple and cost-effective assay for implementation into routine patient care?

This thesis deals with these questions and focuses on the advantages and disadvantages of PCR-based MRD detection in particular.

Pediatric ALL, prognostic factors and treatment

Leukemia is characterized by a clonal expansion of an immature lymphoid or myeloid progenitor cell in the bone marrow (BM), leading to an ongoing proliferation and a lack of further differentiation of this malignant clone. As a consequence, the whole BM becomes invaded by the expansion of the leukemic clone. Acute lymphoid leukemia (ALL) is the most prevalent type of leukemia among children and occurs in approximately 85% of leukemia patients. The majority of ALL patients suffers from precursor B-lineage ALL (80 to 85%) whereas 15-20% of patients have T-lineage ALL. The first aim of treatment is to achieve a complete remission. Treatment of ALL has improved in the early 1960s by introduction of combination chemotherapy and in the early 1970s by prophylactic treatment
of the central nervous system (CNS), leading to a better outcome. At present over 80% of children achieve long-term survival and cure. Treatment regimens generally consist of four phases, i.e. induction, consolidation, intensification and maintenance; duration of treatment is two years. Treatment can differ for the various types of leukemia. For instance, specific protocols have been designed for T-ALL,\textsuperscript{13,14} patients with mature B-ALL are treated differently from the B-precursor ALL patients as well. In the Netherlands, the treatment protocols are initiated and studied by the Dutch Childhood Leukemia Study Group (DCLSG). Initially, DCLSG treatment protocols (until protocol ALL-6, 1984)\textsuperscript{15,16} were based on protocols of St Jude Children’s Research Hospital; since 1988, protocols ALL-7 and ALL-8 have a Berlin-Franfurt-Münster (BFM) backbone.\textsuperscript{13,17} Protocol ALL-9 has started in 1997 and is still ongoing. Currently the most commonly used drugs for treatment of ALL are combinations of corticosteroids, vincristine, L-asparaginase, anthracyclines, cyclophosphamide, 6-mercaptopurine, methotrexate and cytarabine.

The selection, schedule and dose of drugs depends on the patient’s risk group. Initially, diagnosis of ALL is based on morphological and cytochemical characteristics of malignant cells in the BM and peripheral blood, supported by immunophenotype analysis. Subsequently, in protocol ALL-9 two patient groups are delineated: the non-high risk (ALL-NHR) and high risk (ALL-HR) group patients. Current prognostic parameters for increased risk of relapse are initial leucocyte count, extramedullary involvement, the presence of specific translocations and immunophenotype. The high-risk patients receive a more intensive regimen. Protocol ALL-6 showed that high dose intravenous methotrexate is as effective as irradiation to prevent CNS relapse and that in standard risk patients anthracycline treatment is not needed. Omitting cranial irradiation in the treatment of the majority of children with ALL has reduced long-term side-effects without jeopardizing the overall cure. The currently used protocol ALL-9 is quite similar to protocol ALL-6.\textsuperscript{18}

**Prediction of outcome in ALL**

A number of clinical and biologic features at initial diagnosis can be used to estimate the relapse hazard in patients, but none is fully indicative for a high relapse risk. The major problem is the uncertainty of whether a complete eradication of the malignant cells has been achieved. New techniques can detect remaining leukemic cells in patients, who by traditional criteria, appear to be in complete remission. The traditional approach using light microscopy for detection of leukemic cells has a detection limit of 5% of leukemic cells among normal cells. Therefore, other techniques are needed. The response of leukemic cells to therapy can be predicted in vivo by measuring the kinetics of MRD. It is also possible to
perform analysis of the cellular response of leukemic cells using in vitro drug resistance assays.

In vitro drug sensitivity testing may give more information on the cellular response of leukemic cells to drugs used for treatment. Cellular drug resistance is thought to be an important cause of induction failure and relapse. Some groups have developed a chemosensitivity assay that uses methyl-thiazol-tetrazolium (MTT) dyes to select effective drugs. The MTT assay relies on the demonstration of living cells by the ability to convert the soluble MTT to insoluble, colored formazan crystals, which is quantitated using a spectrophotometer. It is a short-term assay, using very low number of cells in suspension and giving results in a few days. In vitro drug resistance profiles of different leukemia types reflect the empirically known clinical effectiveness of the different drugs in these subtypes. Results of the MTT assay are related with ultimate outcome in ALL and AML. Disadvantage is that this assay is not routinuously performed and only a few laboratories are experienced in the application of this assay. All studies have been done retrospectively and recently first prospective studies have started.

Previous reports have shown that the detection of MRD at the end of induction is a strong prognostic factor. The most promising methods for detection of MRD are PCR-amplification of leukemia-specific antigen receptor junctional regions and/or chromosomal fusion transcripts, and flow cytometric identification of leukemia associated phenotypes (detecting aberrant protein-expression by ALL cells). Both techniques can detect one leukemic cell among $10^{-3}$ to $10^{-5}$ normal bone marrow mononuclear cells. Immunologic methods, using flow cytometry, are faster and better suited for quantitative measurement, although less often applicable in comparison with the PCR techniques. In these assays, leukemia-associated immunophenotypes are determined by multiparameter flow cytometry, using various combinations of monoclonal antibodies conjugated to different fluorochromes. For each case, marker combinations allowing the detection of one leukemic cell among $10^3$ or more normal nucleated bone marrow cells are selected at diagnosis and applied during clinical remission. Results with four-colour flow cytometry are promising (sensitivity up to $10^{-4}$) and the possibilities to use more combinations will enable to apply this technique in up to 90% of patients.
Detection and quantification of MRD by PCR

Amplification of antigen receptor genes in a polymerase chain reaction (PCR) is increasingly used for detection of MRD.\textsuperscript{3-5,7,9,30} Since the junctional regions of immunoglobulin heavy chain genes (IGH) and T-cell receptor (TCR) genes are rearranged in almost all ALL patients, this PCR-based analysis is applicable in almost all cases of childhood ALL. The antigen-receptor genes comprise several discontinuous germline segments (i.e. V, variable; D, diversity; and J, joining) that undergo rearrangements in lymphoid cells. The uniqueness of the rearrangements derives from the use of one of the many gene segments available from each pool of the V, D, and J gene segments, and from the variability at the recombination site caused by the insertion and deletion of germline nucleotides and insertion of non-template-derived nucleotides (“N-regions”), a reaction mediated by terminal deoxynucleotidy l transferase (TdT).\textsuperscript{7,25} Because such rearrangements are clonal (unique for an individual cell and its progeny), analysis of Ig and TCR gene configurations can be used to track the persistence of malignant clones whose rearrangements were determined at diagnosis (figure 1).

Secondly, it is possible to use fusion gene transcripts of chromosomal aberrations as a stable PCR-target, although most chromosomal aberrations are present in a limited

![Diagram of Immunoglobulin Gene Rearrangement](image)

**Figure 1. Rearrangement of the Immunoglobulin Gene.** Rearrangement of V, D and G genes with incorporation and deletion of nucleotides (n) leads to a patient-specific junctional region. The forward primer is designed on this region and therefore a unique marker for the leukemia in this patient.
number of patients.\textsuperscript{31} Recently, the t(12;21)(p13;q22) translocation with the TEL-AML1 fusion gene, has been described as the most common genetic anomaly in childhood ALL (present in 25\% of the cases). It was also suggested that this translocation identifies a specific clinical subgroup of pediatric ALL patients aged between 1 and 10 years, with B-precursor immunophenotype and non-hyperdiploid karyotype.\textsuperscript{32-34} The prognosis for TEL-AML1-positive patients was shown to be significantly better than for patients without this fusion gene transcript.

Although the PCR technique can reach a sensitivity of 1 malignant cell per $10^5$ normal cells, a major drawback of antigen receptor based PCR is that clonal evolution may hamper detection of MRD. Ongoing rearrangements of IGH and/or TCR genes during disease might result in the loss of junctional regions initially identified at diagnosis, which can lead to false negative results in PCR analysis.\textsuperscript{35-36} There is some evidence that oligoclonality may be associated with a poor prognosis in childhood ALL.\textsuperscript{35,36}

Previous studies have clearly shown that there is a significant association between the MRD detection at the end of induction therapy and outcome.\textsuperscript{3,5,7,9,30} Precise determination of MRD at early remission time-points can be used for discrimination between high- and low-risk patients. Moreover, it has been shown that estimation of the amount of residual disease is important, rather then establishing its presence alone. Until two years ago, most PCR techniques were on semi-quantitative base, e.g. by dot blot hybridisation. The European Organization for Research and Treatment of Cancer (EORTC) group applied a semi-quantitative competitive PCR approach using an internal standard.\textsuperscript{9} Limiting dilution assay (LDA)\textsuperscript{4,37} allows more accurate quantification, but its applicability is limited since it is an extreme laborious technique.\textsuperscript{38,39} The principle of limiting dilution is based on the use of a qualitative all-or-none end point and on the premise that one or more targets in the PCR reaction mixture give rise to a positive end point. Accurate quantification is achieved by performing multiple replicates at serial dilutions of the material to be assessed. At the limit of dilution, where some end points are positive and some are negative, the number of targets present can be calculated from the proportion of positive end points using Poisson statistics. Thus quantification of malignant cells is possible. The Real-Time Quantitative PCR (RQ-PCR) technology circumvents the problem of a too laborious technique. The RQ-PCR measures the amount of specific PCR products during each cycle of the PCR reaction, allowing precise quantification of the original PCR target as compared to control genes.\textsuperscript{40,41} This method is based on the 5'-3' nuclease activity of Taq DNA Polymerase and an internal dual-labeled fluorogenic probe with a 5'-'reporter dye and a 3'-quencher dye. During RQ-PCR, the 5'-3' nuclease activity of Taq DNA polymerase cleaves the hybridized probe and thereby separates the reporter dye from the quencher dye, enabling emission of a fluorescent signal which increases after
Figure 2. Principle of RQ-PCR. The dual-labeled fluorogenic probe consists of a 5'-reporter dye (R) and a 3'-quencher dye (Q). During RQ-PCR, the 5'-3' nuclease activity of Taq DNA Polymerase cleaves the hybridized probe and separates the reporter dye from the quencher dye, enabling emission of a fluorescent signal. This signal increases after each subsequent PCR cycle and is therefore a value for the amount of PCR product.

Each subsequent PCR cycle (figure 2). The real-time detection of fluorescence intensity provides quantitative data, which are based on the early cycles when the fidelity of PCR amplification is the highest. This new technique is, in contrast to the LDA, relatively simple and fast. Recent studies, comparing results of RQ-PCR with LDA, showed that both approaches give comparable results.\textsuperscript{41,42} The initial RQ-PCR used junctional region-specific TaqMan probes, i.e. specific for each patient, which were combined with two germline primers. This approach is rather expensive. Recently it was shown that at least equally sensitive detection and quantification of MRD can be reached by positioning the TaqMan probe and one of the primers at germline sequences of gene segments in combination with one primer, specific for the type of Ig/TCR gene rearrangement.\textsuperscript{42} This will make this method more cost-effective.
Chapter 1

Scope of the Thesis

This thesis addresses four questions as mentioned before. (1) Is it possible to detect and to quantify MRD in children with ALL and can this add to therapy-stratification and outcome of patients? (2) What is the preferable technical approach? (3) Which time-point(s) is (are) the most informative? (4) And finally, how can MRD detection be implemented into routine patient care?

To investigate whether oligoclonality hampers the application of antigen receptor PCR-based MRD detection during the initial phase of treatment, we monitored the kinetics of different subclones in oligoclonal ALL patients during induction therapy (chapter 2).

The t(12;21)(p12;q23) with the TEL-AML1 fusion gene is the most frequent cytogenetic anomaly in childhood ALL. We investigated how this patient group with TEL-AML1-positivity behaves in MRD analysis and how MRD kinetics differ in patients in continuous complete remission compared to relapsed patients (chapter 3). Since a better prognosis for TEL-AML1-positive patients has been shown in comparison with patients without this translocation, we investigated whether MRD information is still necessary in these patients. On the other hand we observed the presence of this translocation during the first months of treatment and investigated whether the MRD results obtained by RT-PCR of TEL-AML1 transcripts correlated with the clinically validated genomic PCR for IGH and TCR gene rearrangements. By doing this, we studied whether this target is suitable for MRD detection in patients with t(12;21)-positive ALL (chapter 4).

It is still a matter of debate which time-point is the most informative in MRD analysis. Data are scarce on the very early effects of treatment. In order to provide these data, bone marrow samples obtained two weeks after start of induction therapy were analyzed to determine the clearance of leukemic cells during the early phase of induction treatment and to find out whether measurement of MRD levels at this time-point contributes to prediction of outcome (chapter 5).

The clinical response of leukemic cells to chemotherapy can also be measured by in vitro analysis of cellular response of leukemic cells at diagnosis using drug resistance assays. We wondered whether the in vitro analysis by MTT assay and the in vivo determination of kinetics of MRD by PCR are measuring the same cellular response on treatment and as a result were identifying the same patients, or whether a combination of both approaches will further increase the predictive value of each single assay. Thus we analysed patients using both the MTT-assay and PCR-based MRD detection in chapter 6.

In 30% of children with ALL their disease relapse, 5% of these relapses occur in the central nervous system (CNS). In chapter 7, we added PCR analysis to conventional detection techniques, i.e. immunophenotyping and cerebrospinal fluid (CSF)
cytomorphology, in order to investigate whether CSF is suitable for PCR studies and whether early detection of CNS relapse is possible by PCR analysis.

Finally in chapter 8 results are summarized and discussed with respect to the clinical value of MRD detection by PCR, hoping that clinically relevant MRD-based risk group stratification can be achieved. This might give major possibilities for prediction of outcome and improvement of survival in conjunction with a decreased frequency of side effects in the near future, due to a better-tailored regimen for the majority of patients.