PhD Thesis

Investigation of residual tumor cells in pediatric t(12;21) positive acute lymphoblastic leukemia

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List of abbreviations

ALL	acute lymphoblastic leukemia
B-ALL	B-cell ALL
BFM	Berlin-Frankfurt-Münster
cDNA	complementary DNA
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
ETV6	ETS variant gene (formerly TEL)
FCM	flow cytometry
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
G6PDH	glucose-6-phosphate dehydrogenase
iFISH	FISH on interphase nucleus
IG	immunoglobulin
IGH	immunoglobulin heavy chain
IGK	immunoglobulin kappa light chain
Kde	kappa deleting element
LOH	loss of heterozygosity
MNC	mononuclear cell
MRD	minimal residual disease
mRNA	messenger ribonucleic acid
pALL	pediatric ALL
PCR	polymerase chain reaction
RNA	ribonucleic acid
RQ-PCR	real-time quantitative PCR
RQ-RT-PCR	real-time quantitative reverse transcription PCR
RT-PCR	reverse transcription PCR
RUNX1	runt-related transcription factor 1 gene (formerly AML1)
SFM	scanning fluorescence microscopy
TCR	T-cell receptor
TCRD	T-cell receptor delta

1. Introduction

Acute lymphoblastic leukemia (ALL) is the most common pediatric malignancy. The results achieved in recent decades in the treatment of pediatric ALL (pALL) speak for themselves, the less than 10% five-year survival rate experienced in the 1960s has risen these days to 85-90%. In our days the therapeutic goal is not only to improve survival, but also to identify subgroups of patients curable with less aggressive treatment and to define a risk-based therapy.

The development of immunological and molecular techniques allowed the detection of residual disease below the sensitivity level of morphological methods. Minimal residual disease (MRD) is the lowest level of leukemic cells detectable with a certain diagnostic procedure. The method should achieve if possible a sensitivity level of 10^{-4} – 10^{-5}, that means to detect one malignant cell among 10000 or 100000 cells. Analysis of MRD is important not only because it defines therapy in an increasing number of protocols but it also predicts outcome independently of other prognostic factors. The AIEOP-BMF ALL 2000 study involves real-time quantitative PCR (RQ-PCR) analysis of clone-specific gene rearrangements while the BIOMED-1 study includes additionally the flow cytometric immunophenotyping and when there is a certain chromosome translocation the analysis of breakpoint fusion region related chimera mRNA expression by real-time quantitative reverse transcriptase PCR (RQ-RT-PCR) to monitor MRD.

Among various cell based approaches, flow cytometric (FCM) immunophenotyping is a rapid, simple and sensitive method for MRD analysis. It relies on the expression of uncommon or aberrant phenotypes of leukemic cells, which are present in many but by far not all cases of pALL. The immunophenotype used to differentiate leukemic cells should not coincide with any normal lymphoid cells, taking into account that certain immunophenotypes apparently not present in bone marrow cells of healthy individuals, may become visible in actively proliferating bone marrow of leukemia patients after chemotherapy. Another important factor affecting the reliability of FCM is the number of cells available to perform the measurement. If we want to detect 1 leukemic cell among 10000 normal ones, at least 100000 mononuclear cells should be examined, since at least 10 leukemic hits should be present to reliably interpret the results of FCM. The markers used should be expressed stably on leukemic cells, at the same time the investigator should be aware of that gain or loss of certain markers may occur during chemotherapy. Further limitation of this method is that

immunophenotypic shifts may occur during the course of the disease, so preferably at least three or more different leukemia-specific markers should be monitored per patient to prevent false negative results.

Another promising, – however, for MRD analysis not widely used – cell based method is scanning fluorescent microscopy (SFM). The method is briefly, on cells selected based on specific phenotype – after *in situ* labeling and relocalization – investigation of genotype can be performed, thus, virtually the pheno- and genotype of the same object can be investigated. By predefined search criteria, large number of cells can be scanned automatically. First, single and overlapping cells must be separated on the basis of morphometric parameters of nuclei then the autofluorescent objects should be excluded. It is followed by immunophenotyping, thus the subsequent fluorescent *in situ* hybridization (FISH) analysis is carried out on single, not autofluorescent, marker-of-interest positive cells. Through automated scanning of immunofluorescence and FISH signal pattern 10^4 cells can easily be examined without significant hands-on time. Since SFM is an *in situ* cell-based technique suitable for testing both phenotype and genotype, this method was used for MRD detection instead of flow cytometry.

PCR analysis of antigen receptor gene rearrangements used for MRD monitoring is a reliable, accurate method and available in most cases of childhood ALL. Rearranged immunoglobulin (IG) and T-cell receptor (TCR) genes are present in a single copy per cell, thus using real-time PCR technique allows a very precise determination of MRD. The B-cell ALL (B-ALL) carries IG heavy chain (IGH) in more than 90%, TCR-δ (TCRD) in ca. 80%, TCR-γ and IG light chain – kappa deleting element (IGK-Kde) in ca. 50%, TCR-α and TCR-β gene rearrangements in 46% and in 29%, respectively. However, IG and TCR genes might undergo secondary rearrangements leading to oligoclonality, i.e. subclones carrying different clonal IG/TCR gene rearrangements may occur. It is possible that some minor clones are hidden at the time of diagnosis and become detectable and dominant only at a later phase of the disease. Therefore the examination of two or more different rearrangements is recommended. Although in the majority of leukemic patients it is possible to identify more than one marker, in about 30% of cases all of them are not detectable with sufficient sensitivity. Identifying the clone specific region of two or more markers per patient is a relatively costly and time-consuming process.

Leukemic cells can also be distinguished from normal cells in the presence of a specific translocation as the fusion gene could result in the expression of aberrant mRNA. In addition to the high sensitivity of the method further advantage is the close relationship

between the molecular aberration and the leukemic clone, which is independent of cellular changes occurring due to therapy or clonal selection. However, chromosomal translocations are present only in approximately 30-40% of childhood ALL cases. It is important to know that the fusion gene products are only leukemia, and not patient specific, which makes a possible cross-contamination and the subsequent false-positive data very difficult to recognize. A further disadvantage of the method is that the number of leukemic cells cannot be calculated precisely, since the number of transcripts in the leukemic cells can vary from patient to patient even in the same ALL subtypes.

In our research we compared our MRD results measured by cell-based SFM, DNA and RNA based PCR methods. The investigations were conducted on the patient subgroup carrying the most commonly occurring t(12;21)(p13;q22) (*ETV6/RUNX1*) chromosomal translocation in childhood ALL. While earlier it was reported that the *ETV6/RUNX1* translocation positive cases have a favorable outcome, other studies contested this because of the high incidence rate of late relapses. The question arose whether with the cell, DNA and RNA based techniques, i.e. with methods detecting different biological targets, there is any heterogeneity detectable in this group of patients which could explain the discrepancies in the literature.

2. Aims

1. Identification of t(12;21) positive pALL cases among the bone marrow samples sent to the Department of Pathology, University of Pécs, using RT-PCR.
2. Identification of the clonal gene rearrangements and design of clone specific primers for the t(12;21) positive cases.
3. Investigation of the minimal residual disease on the follow-up samples of these patients using
    a. RNA-based RQ-RT-PCR method,
    b. DNA-based RQ-PCR method,
    c. cell-based SFM method.
4. Description of practical difficulties of each step of the procedure illustrated by representative examples.
5. Comparison of the RNA and DNA based MRD results measured by real-time PCR, exploring the causes of possible discrepancies.

6. Comparison of the RNA and DNA based MRD data measured by real-time PCR with the results of cell-based, combined pheno- and genotype examining scanning fluorescent microscopy, exploring the causes of possible differences.
7. Analysis of a patient with multiple subclones.

3. Materials and methods

3.1. Samples

Investigations were conducted on bone marrow samples of pediatric acute lymphoblastic leukemia patients diagnosed in the Pathology Department of the University of Pécs. Our lab received samples from the Department of Pediatrics University of Pécs, Department of Pediatrics University of Szeged, Teaching Hospital Markusovszky Szombathely, and the Borsod-Abaúj-Zemplén County Hospital and University Hospital Velkey László Pediatric Health Care Centre. The diagnosis of leukemia was based on the WHO 2008 diagnostic criteria. In case of a precursor B-ALL, the t(12;21) translocation was screened and patients positive for this rearrangement were further investigated. Beyond the diagnostic bone marrow aspiratum follow-up samples defined by therapeutic protocol arrived on day15 (d15), after the induction period on day33 (d33), at the beginning of Protocol M/consolidation phase, before the delayed re-intensification in month5 (m5) and after the end of the treatment (0.5 year intensive + 1.5 year maintenance therapy). In case of relapse the procedure started from the beginning. All children were treated according to ALL IC-BFM 2002 protocol. Written informed consent was obtained from the children's parents or guardians and the study was conducted according to the Declaration of Helsinki.

3.2. DNA and RNA extraction

Bone marrow mononuclear cells (MNC) were separated by density centrifugation. $5x10^5$ cells were used for cytospin preparations. DNA was isolated from fresh or frozen $1x10^7$ MNCs with QIAamp® DNA Blood Mini Kit according to the manufacturer's protocol. Total RNA was isolated directly from $2x10^7$ MNCs with TRIzol® reagent according to the vendor's recommendation.

3.3. t(12;21) RT-PCR

Reverse transcription and nested PCR was performed using primers and protocols described in the literature. REH cell line harbouring the t(12;21)(p13;q22) was used as positive and K562 as negative control. PCR products were evaluated for clonality by 2% agarose gel electrophoresis.

3.4. t(12;21) RQ-RT-PCR

1 μg RNA of the t(12;21) positive patients was reverse transcribed using 1st Strand cDNA Synthesis Kit for RT-PCR (AMV). cDNA was subsequently amplified in LightCycler™ rapid thermal cycler system. The *ETV6/RUNX1* fusion transcript was detected by using fluorescent hybridization probes. The PCR was accomplished with LightCycler FastStart DNA Master Hybridization Probes according to the manufacturer's protocol. After initial denaturation, a total of 50 cycles of denaturation, annealing and extension were performed, completed with melting curve analysis and cooling. The RNA level was normalized by the level of the human glucose-6-phosphate dehydrogenase (G6PDH) RNA present in the same cDNA using LightCycler-h-G6PDH Housekeeping Gene Set.

3.5. IGH, IGK-Kde and TCRD PCR

Monoclonal IG heavy chain (IGH), IG light chain – kappa deleting element (IGK-Kde) and T-cell receptor-δ (TCRD) gene rearrangements were identified with PCR and heteroduplex analysis using family specific primers. A mixture of periferial blood MNCs from five healthy donors was used as negative, and the REH cell line as positive control, respectively. After initial denaturation, a total of 40 cycles of denaturation, annealing and extension were performed, completed with a final elongation and heteroduplex analysis. The PCR product was size fractionated on polyacrylamide gel electrophoresis and visualized with 0.5 μg/ml ethidium-bromide. In case of clonal gene rearrangements the DNA product was regained from the polyacrilamide gel using GenElute™ Gel Extraction Kit. Fluorescent sequencing was performed with BigDye® Terminator Cycle Sequencing Kit and ABI automated sequencer according to the instructions of the manufacturer. The sequencing was performed in both the forward and the reverse direction to avoid accidental sequencer reading failure.

The immunoglobulin and the TCR genes that belong to the immunoglobulin superfamily genes develop after the so-called V(D)J-recombination which combines one of each germline variable (V), diversity (D) and joining (J) gene segments. During the

V(D)J-recombination the terminal deoxynucleotidyl transferase enzyme adds non-templated nucleotides (N) to the joining D-J, and V-DJ DNA segments, furthermore, addition of germline encoded specific palindromic nucleotides (P) may occur. A clone specific primer can be designed to the unique junctional region of the monoclonal and identical gene rearrangements of the leukemic cells. To identify the clone specific junctional regions the germline V, D, J genes were found using the BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and IMGT (www.imgt.org) online sequence alignment tools. Clone specific primers covering the junctional regions were designed using the Oligo 6 Primer Analysis Software.

3.6. IGH, IGK-Kde and TCRD RQ-PCR

RQ-PCRs were performed with LightCycler™ rapid thermal cycler system with germline reverse primers and probes described in the literature and the forward junctional region specific primers designed by ourselves. The product was detected by fluorescence using TaqMan probes. FAM was chosen as reporter dye at the 5'-end of the probes and TAMRA as the quencher dye at the 3'-end. Normal MNC DNA and sterile water were used as negative controls. After initial denaturation, a total of 50 cycles of denaturation, annealing and extension were performed completed with cooling. Sensitivity was established by making serial 10-fold dilutions of diagnostic DNA into negative control MNC DNA. Sensitivity was defined by the largest, but still detectable dilution. Albumin gene was used to correct quantity and quality. Standard curve was created using 10 fold dilution series from Human Genom Albumin.

3.7. Immunocytochemical preparation

SFM investigations were conducted on cytospin preparations made from $5x10^5$ aliquots of mononuclear cells. Unconjugated mouse anti-CD10 was used as primer antibody, the reaction was developed by biotin-labeled anti-mouse antibody and avidin-FITC. The slides were covered with 0.005 μg/ml Vectashield-DAPI solution.

3.8. Scanning fluorescent microscopy (SFM)

The system used for automated scanning and analysis of slides was composed of a motorized microscope station (Zeiss Axioplan 2ie MOT) with high-resolution monochrome CCD camera. The Metafer 4.0 software was used for digital capturing, processing and cytometric measurements. The microscope was equipped with 100W HBO lamp, DAPI, FITC, SpectrumGreen and SpectrumOrange filters, as well as Zeiss Fluar 10x/0.5 and

Plan-Neofluar 40x/0.75 objectives. For identification of nuclei and CD10 immunofluorescence detection, 10x objectives and the MetaCyte software module were used.

After autofocusing in DAPI counterstain channel, close-fitting, non-overlapping digital images were captured of the total area of cytospin preparations. X and Y coordinates of all objects (suspected cells) were stored for later relocalization. Next, pixel-based mean FITC-CD10 signal intensities were measured within each identified object using fixed integration time (0.58 sec). The pixel intensity was also measured in a control channel (SpectrumOrange) in order to identify and exclude autofluorescent objects. DAPI, FITC and SpectrumOrange images of cell like objects were stored and displayed in a gallery in combined RGB (red-green-blue) format, along with distribution of pixel intensities for both FITC and SpectrumOrange. This allowed the separation of CD10+ cells from CD10- cells and autofluorescent objects, respectively. *ETV6/RUNX1* iFISH signals were evaluated only in single, non-autofluorescent, CD10+ cells.

3.9. Interphase fluorescent *in situ* hybridization (iFISH)

After immunophenotyping, interphase fluorescence *in situ* hybridization (iFISH) was performed on the same sample. Cells were stripped and nuclei were labeled with Vysis LSI *ETV6/RUNX1* dual-color, rearrangement-specific fusion, and extrasignal probe set according to the manufacturer's instructions.

Relocalization of CD10+ cells was based on the previously stored X and Y coordinates and gallery images, using the Metafer 4.0 software. The relocalized nuclei were captured both in SpectrumGreen and SpectrumOrange signal channels using the Isis (In situ imaging system) module. Subsequently, FISH pattern of CD10+ cells was investigated and interpreted. Translocation negative nuclei contained two red and two green signals, whereas in the translocation positive nuclei a fusion (yellow) and an extra signal (red) occurred beside the one green and one red signal representing the alleles not involved in the rearrangement.

On average 27050 cells were analyzed per cytospin preparation. Sensitivity and specificity of the combined immunofluorescence and iFISH method were 98.67% and 99.97%, respectively. To consider a cell population as truly positive, double criteria had to be met: both the CD10+ and the t(12;21)+ cells had to exceed the mean false positivity + 2 S.D. threshold of immunophenotyping (0.21% + 2 x 0.15% = 0.51%) and genotyping by FISH (15.80% + 2 x 0.75% = 17.30%), respectively.

4. Results

4.1. Samples

According to the RT-PCR investigations we identified 14 *ETV6/RUNX1* translocation positive precursor-B-ALL children and the cell, DNA and RNA-based tests were conducted on their follow-up samples. The mean age at diagnosis ranged from 2 to 17 years with an average of 6.07 years, the sex ratio was 1:1. The average follow-up period was 30.5 months (20-38 months). One patient relapsed after 24 months, and another one developed only localized meningeal relapse after 27 months from the onset of the disease.

Diagnostic samples collected before the start of the therapy contained very large number of mononuclear cells, while the MNC number count significantly decreased due to the effect of therapy in follow-up samples. The volume of the submitted materials was also highly variable, which was attributable to the difficulties of bone marrow aspiration. It also occurred that the total number of cells was not sufficient to perform all three tests, in which case we had to set up an order of priority. We considered that optimally for RNA isolation $2x10^7$, for DNA isolation $1x10^7$, but for making citospin preparation far less than these, only $5x10^5$ cells were required. However, the sensitivity of SFM is lower than the sensitivity of the other two methods. Taking all these considerations into account we always made in such a situation an individual decision.

The ratio of the amount of a specific target measured using a particular method at the follow-up time point and that in the untreated sample of the same patient defined the MRD level (this value at diagnosis was 1.0) which was also expressed as tumor dilution (clearance). This allowed comparison of data obtained by the different methods. The tumor dilution values were defined as the negative logarithm of the relative amount of specific target.

4.2. Comparison of the RQ-PCR and RQ-RT-PCR data

We were able to detect clonal rearrangements in all patients by DNA based RQ-PCR procedure. In 6 cases one, in 7 cases two, and in one case three clonal markers could be identified. If RQ-PCR analysis of the same sample with two or more independent DNA markers resulted in different MRD levels, the highest MRD value has been taken into account.

The relative amount of *ETV6-RUNX1* mRNA expression at diagnosis showed a high degree of variability, the average value was 4.59 (range: 0.51–12.77). During comparison of the tumor clearance detected by two different methods, in an individual case discordant result was defined as at least one order of magnitude difference. Two out of 6 cases showed this discrepancy at d15. In both cases RQ-RT-PCR results indicated greater reduction of leukemia and in one of these cases the differences persisted up to the next time-point, too. At d33 differences in 3 out of 12 cases have been noticed; in every case the expression based MRD was lower. In two of these cases the difference remained also at w12. At m5 the differences have been identified in 3 out of 14 cases and again the expression based values referred to higher tumor dilution. Thus, out of 42 comparisons in 10 cases (24%) the clearance of a specific target exhibited difference of at least one order of magnitude always in favor of the RNA data indicating higher dilution, thus lower MRD level.

4.3. Comparison of RQ-PCR, RQ-RT-PCR and SFM data

The percentage of CD10 and FISH positive blasts among the mononuclear bone marrow cells is proved to be on average 82.88% at onset of therapy. Due to the false positive rate ($3x10^{-4}$) and the chosen cut-off value (mean+2 S.D. = 9×10^{-4}) for positivity threshold of the SFM method, the DNA, RNA and cell based results were compared at 10^{-2} and 10^{-3} MRD cut-off levels. By 10^{-2} MRD threshold all patients proved to be negative by all three methods at d33, whereas at 10^{-3} MRD level this was true only for the RNA based approach. At this level and using DNA based analysis all patient reached negativity only by w12. In contrast to the RNA and DNA analysis which indicated MRD negativity at 10^{-3} level for all patients by d33 and w12, respectively, a fraction of patients with residual disease higher than 10^{-3} never dropped to zero by cell based SFM analysis. In addition, by this approach and at this MRD level, the ratio of patients with MRD increased from w12 to m5 significantly (from 13% to 36%), whereas this value remained zero by both RNA and DNA analyses.

This larger fraction of 10^{-3} MRD positive patients by SFM at m5 exhibited on average smaller tumor dilution factor (1.9, range: 1.4 – 2.5) as compared to that (2.1) at w12. Among patients with detectable disease by SFM at m5 two had 1.5 log, the others 2.6 – 3.1 log higher residuum when compared with DNA data, whereas this higher tumor residuum by SFM was in the range of 2.9 – 4.2 order of magnitude as compared with RNA based data. In summary, 1.5 – 4.2 log difference between the amounts of DNA–RNA as well as SFM targets was detected in 5 out 14 patients at m5.

If residual leukemic cells had been detected with SFM method, they should have been detected with RNA and DNA-based methods too, because of the higher sensitivity of PCR-based techniques. However, a preleukemic cell carrying the translocation, but still containing germline antigen receptor gene and not expressing RNA, cannot be detected with PCR-based techniques but with SFM. Based on the FISH signal pattern we have interphase cytogenetic evidence, that the CD10-iFISH positive cells detected by SFM in the 5 patients at w12 and m5 were really precursor leukemic cells and not residual tumor cells. To the development of t(12;21)+ pALL besides the *in utero* monoallelic rearrangement secondary post-natal aberrations are also necessary. This is in 75% of the cases the deletion of the normal *ETV6* gene, resulting in loss of heterozygosity (LOH) or the duplication of the fusion gene. In the untreated samples of the 5 patients complex cytogenetic deviations were showed: in 3 cases rearrangement and normal *ETV6* deletion, in 1 case double fusion gene and in 1 case subclones carrying alternately the two previous variants were identified. In contrast, in the post d33 follow-up samples of these patients in the CD10+ cells only one fusion and normal *ETV6* and *RUNX1* alleles were present, which does not refer to leukemic but leukemia precursor cells.

4.4. Analysis of a patient with multiple subclones

Clone specific DNA based RQ-PCR analyses allowed us to identify a patient with potentially more than one leukemia subclone. In fact, using the multiplex IGH PCR, in 1 out of the 14 patients three clonal IGH gene rearrangements using three different V_H family genes were detected. Sequencing data confirmed this and identified the germline genes as VH3-30, DH6-13, JH4 (IGH1); VH4-31, DH6-13, JH4 (IGH2); VH1-3 DH3-9, JH6 (IGH3). Two out of these three subclones could be justifiably suspected also by flow cytometric phenotyping according to the unique CD10 and CD34 expression of the leukemic cells. During investigation of tumor cell clearance by clone specific DNA based RQ-PCR method, different responses due to the different subclones were observed. Although all three subclones became undetectable by w12 and remained there by m5, different tumor dilution hallmarked by up to 1.4 log difference among the three subclones could be detected at d33. This significant difference among the one and the other two leukemia subclones of this patient might also be existed at later checkpoints (w12 and m5), too, but it was not seen due to the low amount of targets that outranged the sensitivity of our RQ-PCR method.

5. Discussion

Childhood acute lymphoblastic leukemia is treated according to recommendations of international protocols to which the patients are stratified into standard, medium, and high risk groups. The aim of therapy is to further improve the survival, as well as to define risk-based treatment taking into account various prognostic factors, e.g. in case of a high-risk patient a later relapse can be prevented with more aggressive therapy. Age, diagnostic WBC count, early prednisolone response, various structural and numerical chromosomal abnormalities, as well as investigation of minimal residual disease are important in determining prognosis. The major goal of MRD detection is to identify patients who eventually fall into relapse although they belong to good or intermediate prognostic groups, or patients in poor or intermediate risk groups, who could benefit from less intensive treatment.

Currently used strategies for MRD detection rely on the DNA based RQ-PCR analysis of clone-specific gene rearrangements, the RNA based RQ-RT-PCR detection of various chromosome translocation related chimera mRNA expressions and the cell based flow cytometric immunophenotyping. The biological meaning of data obtained by these various approaches as well as their different sensitivity level (10^{-4} to 10^{-5}, 10^{-4} to 10^{-6} and 10^{-3} to 10^{-4}, respectively) might render comparison of data and determination of the method of choice difficult. Furthermore, each technique has advantages and drawbacks, too.

An alternative approach to detect rare malignant events among normal cells is scanning fluorescent microscopy. Since SFM is an *in situ* cell-based technique providing both phenotype and genotype of the cellular target, this method was used for MRD detection instead of flow cytometry. The SFM data were compared with DNA and RNA-based MRD values, a comprehensive study which has not been done, yet.

Our investigations were conducted on t(12;21) translocation positive pALL subgroup that makes up appr. 25% of childhood ALL cases. According to the relevant literature there are only few reports about the comparison of RQ-PCR and RQ-RT-PCR data monitoring MRD in *ETV6/RUNX1* positive patients. Discrepancies of more than one or even two orders of magnitude between DNA and RNA based MRD levels have been described by some authors.

In our studies we investigated 63 bone marrow samples of 14 *ETV6/RUNX1* positive patient with DNA and RNA based PCR and *in situ* cell-based SFM methods. The ratio of the amount of a specific target obtained by a particular method at the different follow-up time

points and in the untreated sample of the same patient defined the MRD level which was also expressed as tumor dilution. This allowed comparison of data obtained by the different methods. Having analyzed MRD data we have ended up with three observations:

1. Tumor clearance by chimera mRNA is faster.

Tumor clearance by chimera mRNA is faster than tumor clearance with other methods since in all cases when at least one log difference between MRD of DNA and RNA could be detected, the RNA values were always lower. This occurred in significant fraction (appr. in a quarter) of analyses. Furthermore, significantly larger fraction of patients reached an MRD level of 10^{-4} faster according to RNA-based method than with DNA based technique. These findings are not suprising as the RNA results cannot indicate the changes in tumor load under all circumstances, but reflect to the number of a particulate chimera mRNA species in sample, thus rather to the cellular activity. Chimera expression rate is influenced by local and systemic environmental factors, like it may change upon therapy, and even the full chimera gene silencing is well documented. These do not contradict to the observations that increase of chimera mRNA as detected by RQ-RT-PCR may herald progression (relapse, blastic crisis) of a particular disease.

In summary, we detected faster tumor clearance by the RNA-based method than by DNA based technique, thus RQ-PCR monitors much reliably tumor load reduction however, RQ-RT-PCR provide valuable information about tumor cell activity.

2. We detected more than 10^{-3} tumor residuum in a couple of patients using SFM.

The most striking finding is that by the end of the observation period in more than one third of patients tumor residuum above 10^{-3} was detected, i.e. CD10 and *ETV6/RUNX1* rearrangement positive cells could be identified by SFM. These values highly exceeded the results obtained by DNA or RNA techniques. We do not believe that this is due to technical error, as the SFM method for this particular target was carefully adjusted, the results were reproducible and the measured values were reliably beyond the false positive rate (0.0003) of the combined *in situ* pheno- and genotyping method. For the same reason we can rule out that the normal, reactive CD10+ hematogones, that are known to increase significantly in number in the post-chemotherapy period, was identified by SFM. The large differences between SFM and RNA results can be explained by what have been discussed under the first section of this chapter. However, as both the clonal DNA markers as well as the immunofluorescence/FISH

combined method identify tumor residuum on a cell-by-cell basis, the significant difference between DNA and SFM data need further considerations.

According to the literature, retrospective analysis of the umbilical cord blood samples of children who subsequently developed *ETV6/RUNX1* + pALL, the t(12;21) translocation on one allele was identifiable already at birth in a large fraction (77%) of the examined patients. These cells reside in the lymphoid stem cell and pro-B-cell compartment, have germline IG genes and the fusion gene might be silenced similarly to early Ph+ progenitors. Leukemia arises when loss of heterozygosity (LOH) occurs in that region, which is typically detected at the onset of this disease.

We believe that we have detected mainly at m5 by SFM these ancestor, preleukemic cells (and not the original tumor cells) which due to the aforementioned reasons could not be picked up either by RQ-PCR or RQ-RT-PCR. Based on the FISH signal pattern we have interphase cytogenetic evidence, that the with SFM method detected double CD10-iFISH positive cells at the 5 patient at w12 and m5 are really precursor leukemic cells and not residual tumor cells. The untreated samples of the 5 patients showed complex cytogenetic deviation, beside the t(12;21) translocation, deletion of the normal *ETV6*, double fusion gene and combination of these was detectable. In contrast, in the post d33 follow-up samples of these patients in the CD10+ cells only one fusion and normal *ETV6* and *RUNX1* alleles were present, which does not refer to leukemic but leukemia precursor cells.

In addition to the cord blood study, further evidence for the existance of such preleukemic clones is provided by publications presenting that the relapsed *ETV6/RUNX1+* pALL still harbored the original t(12;21)(p13;q22) breakpoint fusion, but exhibited a fully different phenotype, antigen receptor gene rearrangements and/or karyotype. These ancestor, preleukemic cells might be the source of a relapse which typically occurs late in this group of pALL, likely, as it does not develop from a true residuum.

One of our patients who relapsed 24 months after the onset, is among the 5 patients for who we could detect these CD10 and *ETV6/RUNX1+* supposedly ancestor cells by SFM. On the contrary, the other patient who exhibited meningeal, but not bone marrow relapse, developed very likely from local residuum, did not belong to that 5 patients' category. These data might strengthen the significance of the SFM findings.

In summary, only the combined *in situ* immuno- and genotyping analysis completed by SFM was able to detect the CD10 and t(12;21) positive, but antigen receptor gene rearrangement negative cells. We could identify these cells which are likely ancestor

preleukemic cells in 1/3 of the patients in the post-induction period. These cells might be the source of some late relapses in this group of disorder.

3. Detection of leukemic subclones with different biological behavior in one patient.

In one of our patients we could identify multiple leukemia subclones according to the IGH gene variable region analysis. Sequencing of the full V-J regions confirmed the presence of three distinct subclones. None of them has been smoldering background subclone as all three were present in similarly high quantity at the onset of the disease. Two of them behaved similarly and exhibited a significantly smaller tumor clearance than the third one during the induction period. This finding reveals clonal heterogeneity in response to the same therapy.

In summary, different subclones identified with DNA-based method may respond distinctly to treatment, therefore the relationship between the antigen receptor gene rearrangements and response to therapy needs further investigation.

6. Summary of new findings

1. For minimal residual disease (MRD) detection in *ETV6/RUNX1* positive childhood pre-B ALL in addition to the RNA-based RQ-RT-PCR and DNA-based RQ-PCR we developed a combined immunophenotype-iFISH scanning fluorescence microscopy (SFM) method based on consecutive labeling and detection of CD10 (immunofluorescence) and t(12;21) translocation (iFISH). The CD10+/t(12;21)- hematogones can be distinguished from CD10+/t(12;21)+ leukemic cells at $9x10^{-4}$ level, so the leukemic cells can be identified with 100% certainty even below the 10^{-3} level.

2. We are the first who compared minimal residual disease data obtained by DNA and RNA-based real-time PCR techniques with the cell-based scanning fluorescence microscopy results.

3. From d33 of treatment detected MRD level was lower than 10^{-3} measured by quantitative RNA and DNA PCR, as well as with combined and immunophenotyping-iFISH method in follow-up bone marrow samples of *ETV6/RUNX1*+ pre-B pALL patients. However, from d33 in 36% of patients 10^{-3} – 10^{-2} CD10+/t(12;21)+ cells were detectable with SFM. These cells showed monoallelic aberrations, were not detectable with the much more

sensitive RNA based RQ-RT-PCR and clone specific DNA-based RQ-PCR techniques, so they were not residual leukemic cells but leukemia precursor bone marrow cells.

4. We detected and visualized for the first time in treated t(12;21)+ pre-B pALL patients *in utero* originated leukemia precursor cells, detectable only with the newly developed combined immunophenotype-iFISH method. The presence of such precursor cells may relate to relapses, so investigation of them could be predictive for the course of the disease.

5. In one patient we could identify multiple leukemia subclones according to the IGH gene variable region analysis. Our research revealed different clearence and sensitivity of the subclones to chemotherapy. Antigen receptor gene rearrangements and the response to therapy need further investigation in the future.

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9. Publications related to the thesis

Original articles:

1. **László R**, Alpár D, Kajtár B, Lacza Á, Ottófy G, Kiss C, Bartyik K, Nagy K, Pajor L. Detection of early precursors of t(12;21) positive pediatric acute lymphoblastic leukemia during follow-up. *Pediatr Blood Cancer*. 2010, 54(1):158-60. IF: 1,948
2. Alpár D, Kajtár B, Kneif M, Jáksó P, **László R**, Kereskai L, Pajor L. Automated detection of residual leukemic cells by consecutive immunolabeling for CD10 and fluorescence in situ hybridization for ETV6/RUNX1 rearrangement in childhood acute lymphoblastic leukemia. *Cancer Genet Cytogenet*. 2007, 173(1):23-30. IF: 1,559

Citable abstract:

1. **László R**, Alpár D, Kajtár B, Lacza Á, Pajor L. Sejt, gén és expresszió alapú technikák alkalmazása a minimális reziduális betegség nyomonkövetésére. *Hematológia-Transzfuziológia*. 2006, 1. Suppl., 39:35.

Oral presentations related to the topic of the thesis:

1. **László R**, Alpár D, Kajtár B, Lacza Á, Pajor L. Sejt, gén és expresszió alapú technikák alkalmazása a minimális reziduális betegség nyomonkövetésére. Malignus Lymphoma Konferencia. Győr, 2006. ápr. 6-8.
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1. **László R**, Degrell P, Kellermayer M, Bollmann D, Egyed M, Seres L, Pajor L. Crystal-storing histiocytosis associated with only one of two consecutive, but genetically unrelated B-cell lymphomas. *Pathol Res Pract.* 2009, 205(4):273-8. IF: 1,219.
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3. Alpár D, Kajtár B, Tóth J, Nagy Z, Jáksó P, **László R**, Kereskai L, Pajor L. Automated evaluation of dual fusion and breakapart FISH probes on paraffin-embedded tissue sections. *Blood review*. 2007, 1. Suppl., 21:124. IF: 5,922.
4. Kereskai L, Jáksó P, **László R**, Kálmán E, Pajor L. A malignus lymphomák aspirációs cytológiai diagnosztikájának lehetőségei. *Hematológia-Transzfuziológia*. 2006, 1. Suppl., 39:26.

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