Gene rearrangement study for minimal residual disease monitoring in children with acute lymphocytic leukemia

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Objective: *To detect markers for minimal residual disease monitoring based on conventional polymerase chain reaction for immunoglobulin, T-cell receptor rearrangements and the Sil-Tal1 deletion in patients with acute lymphocytic leukemia.*

Methods: *Fifty-nine children with acute lymphocytic leukemia from three institutions in Minas Gerais, Brazil, were prospectively studied. Clonal rearrangements were detected by polymerase chain reaction followed by homo/heteroduplex clonality analysis in DNA samples from diagnostic bone marrow. Follow-up samples were collected on Days 14 and 28-35 of the induction phase. The Kaplan-Meier and multivariate Cox methods were used for survival analysis.*

Results: *Immunoglobulin/T-cell receptor rearrangements were not detected in 5/55 children screened (9.0%). For precursor-B acute lymphocytic leukemia, the most frequent rearrangement was IgH (72.7%), then TCRG (61.4%), and TCRD and IgK (47.7%); for T-acute lymphocytic leukemia, TCRG (80.0%), and TCRD and Sil-Tal deletion (20.0%) were the most common. Minimal residual disease was detected in 35% of the cases on Day 14 and in 22.5% on Day 28-35. Minimal residual disease on Day 28-35, T-acute lymphocytic leukemia, and leukocyte count above 50 x 10^9/L at diagnosis were bad prognostic factors for leukemia-free survival in univariate analysis. Relapse risk for minimal residual disease positive relative to minimal residual disease negative children was 8.5 times higher (95% confidence interval: 1.02-70.7).*

Conclusion: *Immunoglobulin/T-cell receptor rearrangement frequencies were similar to those reported before. Minimal residual disease is an independent prognostic factor for leukemia-free survival, even when based on a non-quantitative technique, but longer follow-ups are needed.*

Keywords*: Precursor cell lymphoblastic leukemia-lymphoma; Neoplasm, residual; Gene rearrangement; Polymerase chain reaction*

Introduction

Event-free survival (EFS) rates in childhood acute lymphoblastic leukemia (ALL) have reached 85% in high-income countries, mainly because of the use of risk-based chemotherapeutic protocols and supportive care. In Brazil, cooperative studies aiming at establishing standardized treatment were initiated in 1980, with the adoption of the first protocol developed by the *Grupo Brasileiro de Tratamento da Leucemia na Infância* (GBTLI). Since then, five other protocols ensued and survival rates have increased dramatically over the years[1,2].

In an attempt to achieve better risk classification, the GBTLI-99 protocol included the evaluation of early response to treatment based on the white blood cell (WBC) count on Day 7 and bone marrow status on Days 14 and 28 in addition to the classical National Cancer Institute (NCI) criteria (age and WBC count at diagnosis). Minimal residual disease (MRD) was not used for patient stratification but was included as a research topic of the protocol. Several prospective studies demonstrated that MRD data constitute the most relevant and independent prognostic information in childhood ALL[3-7]. Results from the GBTLI-99, restricted to three centers in the state of São Paulo, demonstrated a significant association of MRD positivity and worse survival rates, particularly for the final high-risk patient group[8]. As a result, in the ongoing protocol (GBTLI-2009), MRD on Day 15 and at end of induction (Day 35) is considered an obligatory step for risk assessment.

Hence, it is crucial to establish MRD testing in the main leukemia treatment centers of the country. The gold standard techniques for MRD detection are multiparameter 6-color flow cytometric immunophenotyping and real-time quantitative polymerase chain reaction (RQ-PCR) of the immunoglobulin (*Ig*) and T-cell receptor (*TCR*) genes[9]. These methods, however, are expensive, complex, and require considerable technical expertise. Therefore, as an initial strategy, MRD assay based on conventional polymerase chain reaction (PCR) was chosen to detect *Ig* and *TCR* clonal rearrangements. Herein the results from ALL children treated in three hospitals of Minas Gerais, Brazil are described.

Methods

Patients and diagnosis

Fifty-nine patients with acute lymphoblastic leukemia, aged one to 18 years, were treated

Conflict-of-interest disclosure:
The authors declare no competing financial interest

Submitted: 10/1/2012
Accepted: 12/17/2012

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DOI: 10.5581/1516-8484.20130115

at three different treatment centers in Belo Horizonte, Minas Gerais (Brazil), during this research. Samples were collected prospectively at Hospital das Clínicas, *Universidade Federal de Minas Gerais* (UFMG) from January 2010 to January 2012 (n = 36), at Hospital da Baleia/Fundação Benjamin Guimarães (n = 15) and at Santa Casa de Misericórdia de Belo Horizonte (n = 8) from April 2010 to April 2011. Forty-five patients (76.3%) were treated according to the Brazilian GBTLI-99 protocol, while six (10.2%) were treated according to the GBTLI-2009 and eight (13.5%) according to the European AIEOP-95 protocol. The study was approved by the Ethics Committees of the institutions and was based on the Helsinki convention criteria. Guardians gave their written informed consent to participate in the research.

The diagnosis was made by standard morphological analysis and by flow cytometry immunophenotyping. B-lineage ALL included subtypes B-I (HLA-DR$^+$, TdT$^+$, CD19$^+$, CD79$^{+/-}$), BII and BIII (HLA-DR$^+$, TdT$^+$, CD19/CD10$^+$, CD20$^+$), according to the EGIL classification system[10]. B-IV (CD10$^{+/-}$, CD21$^+$, CD79a$^+$, SmIg$^+$) patients were excluded from this study as they are treated with a specific protocol. T-lineage patients (TdT$^+$, CD3$^+$, CD7$^+$) were not subdivided into subtypes in our analysis. Of the 59 patients initially included, 48 (81.4%) had precursor B-cell ALL (pB-ALL - pro-B ALL in one case and common-ALL/pre-B ALL in the remaining forty-seven), and 11 (18.6%) had T cell-derived ALL. The *BCR-ABL* fusion gene was investigated by reverse transcription PCR (RT-PCR) as proposed by van Dongen et al.[11].

Thirty patients were female and 29 were male. Median age was 5.1 years. Twenty-four patients were initially assigned as low-risk group; three of them were relocated to the high-risk group based on slow response criteria. The main clinical and biological characteristics of patients at diagnosis and in the induction phase are depicted in Table 1.

Table 1 - Clinical and biological variables of children with acute lymphoblastic leukemia from Minas Gerais, Brazil

Variable	%
Age (n = 59)	
1-9 years	62.7
≥ 9 years	37.3
White blood cell count at diagnosis (n = 59)	
< 50x109/L	83.1
≥ 50x109/L	16.9
Central nervous system status at diagnosis (n = 47)	
CNS 3	0
CNS1 or 2	100
White blood cell count on Day 7 (n = 46)	
< $5x10^9$/L	80.4
≥ $5x10^9$/L	19.6
Bone marrow on Day 14 (n = 33)	
M1/M2	78.8
M3	21.2
Bone marrow on Day 28-35 (n = 57)	
M1	96.5
M2/M3	3.5
Response (n = 48)	
Good responder	72.9
Poor responder[1]	27.1
Final risk group (n = 59)	
Low risk	35.6
High risk	64.4
Immunophenotype (n = 59)	
Precursor B-lineage	81.4
T-lineage	18.6
***BCR-ABL* fusion transcript (n = 51)**	
Positive	5.9
Negative	94.1
Event at induction (n = 59)	
Complete remission	96.6
Death during induction	3.4

[1]Poor responder: patients who fulfilled one or more of the following criteria during the phase of remission induction: white blood cell count on Day 7 ≥ $5x10^9$/L, bone marrow M3 (> 25% blasts) on D14 or bone marrow M2 or M3 (> 5% and 25% blasts, respectively) on D28/D35.

Cell samples and DNA isolation

Bone marrow samples were obtained from the patients at diagnosis and on Day 14 from the beginning of the treatment and at the end of induction phase (Day 28 for patients treated according to the GBTLI-LLA-99 protocol, Day 33 for those treated by the AIEOP-95 and Day 35 for those treated by the GBTLI-LLA-2009). Mononuclear cells were separated by a Ficoll-Paque centrifugation gradient, and DNA was extracted with QIAamp DNA Blood kit (QIAGEN), according to manufacturer instructions. The extracted DNA was quantified in the NanoDrop 2000™ Spectrophotometer (Thermo Scientific).

PCR amplification at diagnosis

DNA from diagnostic samples was amplified using eight primer mixes according to the ALL subtype[12]. For pB-ALL, one PCR reaction was tested for the *IgH* gene (*FR3A-LJH*)[13,14], two multiplex reactions for *IgK* rearrangements (combinations of Vκ and intron-3 forward primers and the Kde-3 reverse primer)[15], three multiplex reactions for *TCRG* with the primers described by Trainor et al.[14] (combinations of Vγ2/Vγ8, Vγ3/Vγ5, Vγ9 forward primers and Jγ reverse primers as described by Assumpção et al.)[12], and two PCR reactions for *TCRD* incomplete rearrangements (Vδ2-Dδ3 and Dδ2-Dδ3)[15]. For T-lineage ALL, five multiplex reactions were tested for *TCRG*[12], three PCR reactions for *TCRD* rearrangements (Vδ2-Dδ3, Vδ2-Jδ1 and Vδ3-Jδ1)[16], and one PCR reaction for the *Sil-Tal* deletion (primers Sil5´and tal1db1, as described by Pongers-Willemse et al.)[16]. DNA quality of samples that did not amplify in any PCR reaction was assessed through amplification of the *FLT3* gene according to Meshinchi et al.[17].

PCR were carried out in 25 μL reactions containing 50 ng of DNA, 1 U of Tth DNA polymerase (Biotools), 7.5 pmol of each primer, 1.5 mM $MgCl_2$ and 80 μM of each dNTP; PCR amplification cycles have been described previously[8,16]. Two negative controls were used in each PCR assay: one without DNA and the other containing polyclonal DNA obtained from peripheral blood mononuclear cells (PBL) from eight healthy donors.

Homo/Heteroduplex analysis

Homo/heteroduplex analysis was adapted from Langerak et al.[18]. Briefly, 5 µL of sample buffer were added to 20 µL of PCR product, which was then denatured at 95°C for 5 minutes, renatured at 4-8°C for 1 h, and submitted to electrophoresis in a non-denaturant 12% polyacrylamide gel at room temperature. The gel was stained in 3X Sybr Safe® (Life Technologies) for 40 min. An amplified gene rearrangement was characterized as clonal when a band of the expected size was visible, and was not present in the PBL control. The expected fragment sizes are: 80 to 120 base pairs (bp) for *IgH* [13,14], 440 to 608 bp for *TCRD*[16], 170 to 230 bp for *TCRG*[14], 433 to 511 bp for *IgK* mix 1, 429 to 443 bp for *IgK* mix 2[15], and 300 bp for *Sil-Tal1*[16].

PCR amplification on Days 14 and 28-35

For MRD monitoring, at least two clonal markers and primer mixes with the highest sensitivity (*TCRD* and *IgK* = 10^{-3}) were selected whenever possible. PCR reactions were carried out as described above, except that 500 ng of DNA were used for Day 14 and Day 28 samples. Samples from the diagnoses were also tested in the same PCR reaction, as well as the normal PBL and a non-template control. All PCR products were submitted to homo-heteroduplex analysis. Follow-up samples were considered positive when they showed the same migration pattern and molecular weight as the samples from the diagnosis. DNA quality was confirmed through *FLT3* PCR amplification.

Statistical analysis

The Fisher test was used to assess the association between MRD at the time points studied (Day 14 and Day 28-35) and the following clinical and biological categorical variables: age, WBC count at diagnosis, immunophenotype, risk group, type of response and WBC count on Day 7 (for categories, see Table 1). Overall survival (OS) and leukemia-free survival (LFS) curves were plotted according to the Kaplan-Meier method. OS was calculated from the data of diagnosis to the date of death or last follow-up. LFS was calculated from the date of remission (two patients who died before remission were accordingly excluded) to the date of relapse or last follow-up. Two patients who died in complete remission because of infectious complications were censored when they died. Curves for different groups were compared by the log-rank test. All statistical analyses were performed using the Statistical Program for the Social Sciences (SPSS) software version 17.0, with the level of significance set at p-value ≤ 0.05. The prognostic impact of minimal residual disease at Day 28/35 was adjusted for the effect of WBC count at diagnosis and immunophenotype in a multivariate Cox model.

Results

Three out of 59 patients were not tested for gene rearrangements because there was not enough bone marrow material for molecular biology studies at diagnosis. One case showed no amplification in the positive control PCR reaction, so the patient was excluded from the analysis. *Ig/TCR* rearrangements were not detected in five (9%) patients out of 55 screened: 2/44 had pB-ALL and 3/11 were T-ALL patients. Two or more clonal markers were detected in 34 children (61.8%): 30 out of 44 (68.2%) for pB-ALL and four out of 11 (36.4%) for T-ALL.

The most frequent rearrangement for pB-ALL was *IgH* (72.7%), followed by *TCRG* (61.4%) and *TCRD* and *IgK* (47.7%). For T-ALL, the most frequent rearrangement was *TCRG* (80%), followed by *Sil-Tal1* and *TCRD* (20%). Frequencies per PCR reaction are shown in Table 2 (pB-ALL) and Table 3 (T-ALL).

Table 2 - Frequency of *Ig* and *TCR* rearrangements in 44 children with precursor B-lineage acute lymphoblastic leukemia from Minas Gerais, Brazil

Gene Rearrangement	Frequency
IgH	
FR3A - LJH	32/44 (72.7%)
IgK	
VkI - intron, Kde	7/42 (16.3%)
VkII, VkIII - Kde	16/44 (36.4%)
TCRG	
Vγ2, Vγ8 - Jγ1.3/2.3, Jγ1.1/2.1, Jγ1.2	17/44 (38.6%)
Vγ3, Vγ5- Jγ1.3/2.3, Jγ1.1/2.1, Jγ1.2	9/44 (20.5%)
Vγ9 - Jγ1.3/2.3, Jγ1.1/2.1, Jγ1.2	11/44 (25%)
TCRD	
V*d*2 - D*d*3	20/44 (45.5%)
D*d*2 - D*d*3	4/43 (9.3%)

Table 3 - Frequency of *TCR* rearrangements and *Sil-Tal* deletions in 11 children with T-lineage acute lymphoblastic leukemia from Minas Gerais, Brazil

Gene Rearrangement	Frequency
TCRG	
Vγ2, Vγ8 - Jγ1.3/2.3, Jγ1.1/2.1, Jγ1.2	6/11 (54.6%)
Vγ3, Vγ5- Jγ1.3/2.3, Jγ1.1/2.1, Jγ1.2	2/11 (18.2%)
Vγ9 - Jγ1.3/2.3, Jγ1.1/2.1, Jγ1.2	0/10 (0%)
Vγ10 - Jγ1.3/2.3, Jγ1.1/2.1, Jγ1.2	1/10 (10%)
Vγ11 - Jγ1.3/2.3, Jγ1.1/2.1, Jγ1.2	1/10 (10%)
TCRD	
V*d*2 - D*d*3	0/11 (0%)
V*d*2 - J*d*1	0/10 (0%)
V*d*3 - J*d*1	
Sil-Tal	2/10 (20%)
Sil-Tal1	2/10 (20%)

Table 4 - Minimal residual disease (MRD) data on Days 14 and 28-35 of the induction phase in children with acute lymphoblastic leukemia from Minas Gerais, Brazil

Variable	Percentage
MRD Day 14 (n = 29)	
Negative	19 (65.5%)
Positive	10 (34.5%)
MRD Day 28-35 (n = 40)	
Negative	31 (77.5%)
Positive	9 (22.5%)
MRD Day 14 and Day 28-35 (n = 24)	
Negative Day 14 and Day 28	15 (60%)
Positive Day 14 and negative Day 28	5 (20%)
Positive Day 14 and Day 28	4 (16%)

Table 5 - Cox's model for the prognostic influence of minimal residual disease on Day 28-35 on the leukemia-free survival of children with acute lymphoblastic leukemia from Minas Gerais, Brazil

Variable	B coefficient	Standard error	p-value	Relative risk[1]	
				Estimated	95% CI
Immunophenotype	1.577	1.07	0.14	4.84	0.59-39.58
WBC[2] at diagnosis	1.588	0.99	0.11	4.89	0.70-34.03
MRD[3] on Day 28-35	2.141	1.08	0.048	8.51	1.02-70.69

95% CI: 95% confidence interval

[1] Relative risk refers to leukemia relapse in T-acute lymphocytic leukemia relative to precursor-B acute lymphocytic leukemia

[2] White blood cells (WBC) ≥ 50 x 10^9/L relative to WBC < 50 x 10^9/L

[3] Positive minimal residual disease (MRD) relative to negative MRD

Follow-up bone marrow samples for MRD monitoring were available for 44 patients. They were drawn on Day 14 in 29 cases, and on Day 28-35 in 40 cases. Twenty-four patients had samples from both times. As shown in Table 4, positive MRD was detected in 34.5% of the cases on Day 14 (95% confidence interval – 95% CI: 19.6-49.4) and in 22.5% on Day 28-35 (95% CI: 9.6-35.4). Eight out of nine patients who were positive for MRD on Day 28 had pB-ALL.

Of the 54 follow-up samples that were tested for more than one clonal marker, nine (16.7%) had discrepancies between different PCR reaction results, most likely because of the different sensitivities between PCR mixes[12]. These discrepancies were mainly detected in samples positive for *TCRD* and negative for *TCRG* (three cases), positive for *TCRD* and negative for *IgH* (two cases), and positive for *IgK* and negative for *IgH* (two cases).

MRD results were tested for patients grouped according to clinical and laboratorial variables. The only statistically significant association found was between age at diagnosis and MRD at Day 28-35. For some variables it was impossible to test associations since some categories had a low number of patients (i.e. central nervous system stages 2 or 3 at diagnosis, bone marrow stages 2 or 3 at Day 28-35).

The median follow-up time of patients who did not have an event was 20 months. In only one case there was loss of follow-up after 15 months. This patient was in remission and was censored at that point. The 2-year probability of overall survival was 84.8% ± 5.0% (SE), while the 2-year probability of leukemia-free survival was 73.8% ± 8.8% (Figure 1A). The 2-year LFS was considerably lower in the T-ALL group (43.8% ± 17.6%) when compared to the pB-ALL group (83.2 ± 9.5%; p-value = 0.005), and significantly lower in the group of patients with WBC count at diagnosis above 50 x 10^9/L (19.4% ± 17.2%) than in the group with WBC count below 50 x 10^9/L (84.4 ± 8.8%; p-value = 0.0002).

Positive MRD on Day 28-35 was associated with a lower probability of LFS, but not significantly, in univariate analysis (p-value = 0.19; Figure 1B). When the prognostic impact of minimal residual disease at Day 28-35 was adjusted for the effect of WBC count at diagnosis and immunophenotype in a multivariate Cox model, MRD was the most significant factor (p-value = 0.048; Table 5). The probability of relapse for positive DRM patients on Day 28-35 was 8.5 greater than for those with negative DRM (95% CI: 1.02-70.69).

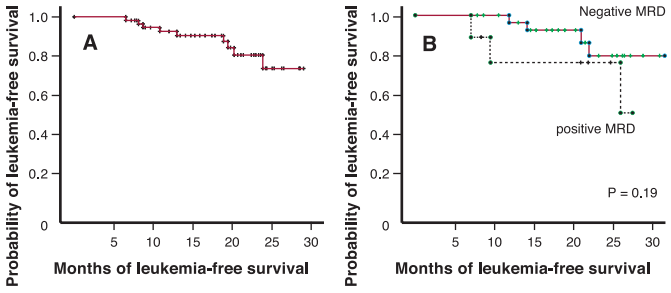

Figure 1 – A: Leukemia-free survival for all patients (n = 57); B: Leukemia-free survival for patients with positive (n = 9) or negative (n = 31) minimal residual disease (MRD).

Discussion

A low cost methodology to detect markers for MRD monitoring based on conventional PCR for *Ig* and *TCR* rearrangements and *Sil-Ta11* deletion was investigated. In pB-ALL patients, the prevalence of rearrangements for the *IgH* (71%), *IgK* (47%), *TCRG* (60%) and *TCRD* (47%) genes was quite similar to those found in previous Brazilian studies using a similar methodology[8,12,19]. For T-ALL, *TCRG* prevalence was lower (80%) when compared to other studies: 98%-100%[8,12]. It is intriguing that V9 rearrangements were absent in T-ALL patients in the present study. The *Sil-Tal*1 rearrangement was found in 20% of T-ALL cases, in agreement with another Brazilian study[20].

At least one clonal rearrangement was detected at diagnosis in most patients (91%), although several T-ALL patients had no markers identified (27.3%), which shows that additional genes, such as *TCRβ*, must be screened in this group of patients. Two or more clonal rearrangements were detected in 68.2% of pB-ALL cases and in 36.4% of T-ALL patients. MRD assays usually require two independent PCR reactions because some rearrangements are unstable and may undergo changes during the course of treatment[21,22]. Although it is unlikely that clonal evolution occurs during the one-month period of remission induction, it is still possible that a minor clone with a different *Ig/TCR* rearrangement ends up being selected during the course of chemotherapy. Therefore, it is advisable that residual leukemic cells, whenever possible, are monitored through two or more rearrangements, choosing primer mixes with the highest sensitivity.

MRD was positive on Day 14 in 34.5% of the cases and on Day 28 in 22.5%. The percentages of positive cases in Minas Gerais were somewhat higher than the 26% and 13%, respectively, previously reported in São Paulo[8]. One possible explanation for this discrepancy is that in previous reports[8] mainly retrospective samples were included. Frozen cell pellets in our experience give rise to lower quality DNA in terms of sensitivity reached by MRD analysis. In the ongoing prospective study conducted at the Boldrini Children's Center (São Paulo), the percentage of MRD-positive patients at Day 35 is 27% (Ganazza M., personal communication).

MRD on Day 28-35 was the most significant prognostic factor for leukemia-free survival when adjusted for the effect of WBC count at diagnosis and immunophenotype. The probability of relapse for MRD-positive was 8.5 greater than that for MRD-negative patients. Caution in interpreting this finding is, however, strongly recommended, because the number of patients in the present study was rather low, as reflected in large 95% CI limits (1.02-70.7).

Most patients who were positive for MRD on Day 28-35 had pB-ALL (eight out of nine). The analysis of T-ALL cases by conventional PCR may be hampered by the low sensitivity of the PCR multiplex mix used to detect the most frequent target (*TCRG*) [12] and by the low frequency of patients with more than one clonal target. New primer duplexes are being tested to improve sensitivity and target availability for MRD testing in these cases.

Although the methodology chosen to evaluate MRD in this study has a low sensitivity, it is sensitive enough to detect patients with a high load of tumor cells at the end of induction. In our study, the frequency of MRD on Days 28-35 was higher among patients classified as high risk (78%). Scrideli et al.[8] demonstrated that within the high-risk group, the 5-year EFS was significantly associated with MRD on Day 28 (MRD negative: 80.1%; MRD positive: 23.7%; p-value < 0.00001).

Conclusion

MRD disease information may be very informative for indicating early bone marrow transplantation or intensification of chemotherapy for high-risk patients who turn out to be positive at the end of the induction phase.

We are currently comparing the results described herein with quantitative real-time PCR analysis using specific primers for the V-D junction and TaqMan probes, aiming to understand how well the two techniques correlate, and whether the conventional PCR may be used in the context of the current GBTLI protocol. As this is a prospective study, those matters will be addressed as soon as we reach a longer follow-up time.

Acknowledgments

We thank all research subjects and their families for their kind cooperation. The authors also thank Valéria Cristina Câmara (Laboratório Central, Hospital das Clínicas, UFMG) for technical assistance; Dr. José Andres Yunes (Centro Infantil Boldrini) for revision of this manuscript; and health care professionals from the participant institutions, especially Dr Cybele de Andrade Paes. This study was supported by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (Fapemig).

References

1. Brandalise S, Odone V, Pereira W, Andrea M, Zanichelli M, Aranega V.,. Treatment results of three consecutive Brazilian cooperative childhood ALL protocols: GBTLI-80, GBTLI-82 and -85. ALL Brazilian Group. Leukemia. 1993;7(Suppl 2):S142-5.
2. Brandalise SR, Pinheiro VR, Aguiar SS, Matsuda EI, Otubo R, Yunes JA, et al. Benefits of the intermittent use of 6-mercaptopurine and methotrexate in maintenance treatment for low-risk acute lymphoblastic leukemia in children: randomized trial from the Brazilian Childhood Cooperative Group--protocol ALL-99. J Clin Oncol. 2010;28(11):1911-8.
3. Jacquy D, Delepaut B, Van Daele S, Vaerman JL, Zenebergh A, Brichard B, et al. A prospective study of minimal residual disease in childhood B-lineage acute lymphoblastic leukaemia: MRD level at the end of induction is a strong predictive factor of relapse. Br J Haematol. 1997;98(1):140-6.
4. Cavé H, van der Werff ten Bosch J, Suciu S, Guidal C, Waterkeyn C, Otten J, et al. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia. European Organization for Research and Treatment of Cancer-Childhood Leukemia Cooperative Group. N Engl J Med. 1998;339(9):591-8. Comment in: N Engl J Med. 1998;339(9):627-9; N Engl J Med. 1999;340(2):152-3; author reply 153-4; N Engl J Med. 1999; 340(2):153-4.
5. van Dongen JJ, Seriu T, Panzer-Grümayer ER, Biondi A, Pongers-Willemse MJ, Corral L, et al. Prognostic value of minimal residual disease in acute lymphoblastic leukaemia in childhood. Lancet. 1998;352(9142):1731-8. Comment in: Lancet. 1999; 353(9154):752-3.
6. Coustan-Smith E, Behm FG, Sanchez J, Boyett JM, Hancock ML, Raimondi SC, et al. Immunological detection of minimal residual disease in children with acute lymphoblastic leukaemia. Lancet. 1998;351(9102):550-4. Comment in: Lancet. 1998;351(9111):1287.
7. Panzer-Grümayer ER, Schneider M, Panzer S, Fasching K, Gadner H. Rapid molecular response during early induction chemotherapy predicts a good outcome in childhood acute lymphoblastic leukemia. Blood. 2000;95(3):790-4. Erratum in: Blood. 2000;95(100):3010. Comment in: Blood. 2000;96(4):1619-20.
8. Scrideli CA, Assumpção JG, Ganazza MA, Araújo M, Toledo SR, Lee ML, et al. A simplified minimal residual disease polymerase chain reaction method at early treatment points can stratify children with acute lymphoblastic leukemia into good and poor outcome groups. Haematologica. 2009;94(6):781-9.
9. Cazzaniga G, Biondi A. Molecular monitoring of childhood acute lymphoblastic leukemia using antigen receptor gene rearrangements and quantitative polymerase chain reaction technology. Haematologica. 2005:90(3):382-90.
10. Bene MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A, et al. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). Leukemia. 1995;9(10):1783-6. Comment in: Leukemia. 2001;15(10):1673-4.
11. van Dongen JJ, Macintyre EA, Gabert JA, Delabesse E, Rossi V, Saglio G, et al. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. Leukemia. 1999;13(12):1901-28.
12. Assumpção JG, Ganazza MA, de Araújo M, Silva AS, Scrideli CA, Brandalise SR, et al. Detection of clonal immunoglobulin and T-cell receptor gene rearrangements in childhood acute lymphoblastic leukemia using a low-cost PCR strategy. Pediatr Blood Cancer. 2010;55(7):1278-86.
13. Trainor KJ, Brisco MJ, Story CJ, Morley AA. Monoclonality in

B-lymphoproliferative disorders detected at the DNA level. Blood. 1990;75(11):2220-2.

14. Trainor KJ, Brisco MJ, Wan JH, Neoh S, Grist S, Morley AA. Gene rearrangement in B- and T-lymphoproliferative disease detected by the polymerase chain reaction. Blood. 1991;78(1):192-6. Comment in: Blood. 1992;79(1):291-2.
15. Nakao M, Janssen JW, Bartram CR. Duplex PCR facilitates the identification of immunoglobulin kappa (IGK) gene rearrangements in acute lymphoblastic leukemia. Leukemia. 2000;14(1):218-9.
16. Pongers-Willemse MJ, Seriu T, Stolz F, d'Aniello E, Gameiro P, Pisa P et al. Primers and protocols for standardized detection of minimal residual disease in acute lymphoblastic leukemia using immunoglobulin and T cell receptor gene rearrangements and TAL1 deletions as PCR targets: report of the Biomed-1 Concerted Action: investigation of minimal residual disease in acute leukemia. Leukemia. 1999;13(1):110-8. Comment in: Leukemia. 2001;15(8):1301-3.
17. Meshinchi S, Woods WG, Stirewalt DL, Sweetser DA, Buckley JD, Tjoa TK, et al. Prevalence and prognostic significance of Flt3 internal tandem duplication in pediatric acute myeloid leukemia. Blood. 2001;97(1):89-94.
18. Langerak AW, Szczepański T, van der Burg M, Wolvers-Tettero IL, van Dongen JJ. Heteroduplex PCR analysis of rearranged T cell receptor genes for clonality assessment in suspect T cell proliferations. Leukemia. 1997;11(12):2192-9.
19. Ganazza MA, Assumpção JG, de Araújo M, Scrideli CA, Tone LG, Brandalise SR et al. TCRG gene rearrangement patterns in brazilian children with ALL: An update. Leuk Res 2009:33(12):228-9.
20. Mansur MB, Emerenciano M, Brewer L, Sant'Ana M, Mendonça N, Thuler LC et al. SIL-TAL1 fusion gene negative impact in T-cell acute lymphoblastic leukemia outcome. Leuk Lymphoma 2009; 50(8):1318-25.
21. Wasserman R, Yamada M, Ito Y, Finger LR, Reichard BA, Shane S et al. VH gene rearrangement events can modify the immunoglobulin heavy chain during progression of B-lineage acute lymphoblastic leukemia. Blood. 1992;79(1):223-8.
22. Guggemos A, Eckert C, Szczepanski T, Hanel C, Taube T, van der Velden VH et al. Assessment of clonal stability of minimal residual disease targets between 1st and 2nd relapse of childhood precursor B-cell acute lymphoblastic leukemia. Haematologica. 2003;88(7):737-46.

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