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

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EARLY DETECTION OF CENTRAL NERVOUS SYSTEM RELAPSE BY PCR IN CHILDREN WITH B-PRECURSOR ACUTE LYMPHOBLASTIC LEUKEMIA

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Early detection of central nervous system relapse by PCR in children with B-precuror Acute Lymphoblastic Leukemia

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ABSTRACT

Still five percent of the relapses in children with childhood B-precuror acute lymphoblastic leukemia (ALL) occur in the central nervous system (CNS), which confers a poor prognosis. Detection of ALL blasts in the CNS is routinely performed by morphological examination and immunophenotyping. Previously, it has been shown that the PCR technique is a useful and sensitive method to detect minimal residual disease (MRD) in bone marrow (BM).

In the present study we investigated whether PCR can be applied on cerebrospinal fluid (CSF) samples to detect CNS involvement. Fifteen selected patients with ALL were studied. Patient-specific nested PCRs for immunoglobulin heavy chain (IGH) and T-cell-receptor (TCR) gene rearrangements were performed on simultaneously collected BM and CSF samples, which were consecutively taken at diagnosis and during follow-up. In the patients that relapsed in the CNS (n=5), MRD was detected in the CSF at least four weeks before the diagnosis of CNS relapse could be made by standard techniques. Furthermore, in all cases, PCR-positivity of CSF was followed by overt CNS relapse. In four out of five cases, residual disease in the bone marrow was absent at the moment of isolated CNS relapse. The five patients that had relapsed in the BM, and the five patients that remained in CCR (follow-up 95 months) were negative by PCR analysis for leukemic cells in all CSF samples.

Detection of MRD in CSF by PCR is a sensitive method for early detection of leukemic cells in the CSF. MRD analysis by antigen receptor PCR assays is nowadays routinely performed on blood and bone marrow samples; our study shows that this can eventually be expanded with analysis of CSF.
INTRODUCTION

In children with B-precursor acute lymphoblastic leukemia (ALL) the overall survival is over 70%. Despite the success of current treatment, thirty percent of the children experience a relapse.\(^1\) Although intensive systemic chemotherapeutic regimens incorporating CNS prophylaxis have decreased the incidence of central nervous system (CNS) relapse,\(^17\) five percent of the relapses still occur in the CNS. CNS involvement is a life-threatening event. Early detection and treatment of CNS relapse are important with respect to ultimate outcome; therefore, it is important to diagnose CNS involvement as early as possible.\(^2,10^1,10^2\)

Nowadays, detection of ALL blasts in the CNS is routinely performed by morphological examination of cytospins and immunophenotyping. CNS-leukemia is confirmed if the cell number in the CSF is \(>5\) cells per mm\(^3\) and lymphoblasts are evident on the cytocentrifuged specimen. A diagnostic dilemma arises when the CSF cell count is low, and when the interpretation of morphologic abnormalities of CSF cells in cytocentrifuge preparations is cumbersome.\(^13,10^3-10^5\) Often the CSF contains a few morphologically suspect cells already several weeks before CNS relapse. As a result, CNS involvement, although early suspected, is difficult to confirm and false negativity up to 40% for first lumbar puncture has been reported.\(^10^6,10^7\) To differentiate leukemic from normal lymphocytes in the CSF on a cytospin preparation, the use of staining for terminal deoxynucleotidyl transferase (TdT) is helpful.\(^10^8,10^9\) Immunophenotyping techniques are well suited for routine investigation of residual disease because of their rapidity and the high number of cells tested as compared to morphology.\(^28,11^0-11^3\) But this immunological technique is in some cases hampered due to the expression of markers on non-malignant cells. With the antigen-receptor-based PCR assays for the detection of minimal residual disease, it is possible to detect small numbers of malignant cells (up to \(1.10^5\)), not detectable with conventional techniques.\(^3-5,7\) So far, only a few studies have been described in which PCR analysis of CSF samples is performed in patients with ALL at the time of CNS relapse.\(^11^4-11^7\) In none of these studies consecutively and simultaneously taken BM and CSF samples have been analyzed.

In the present study we added PCR analysis to immunophenotyping and CSF morphology, to investigate whether early detection of CNS relapse is possible by PCR analysis. In this report, the following issues to be addressed are: (1) Is CSF suitable for PCR studies; (2) Does detection of MRD in the CSF indicate CNS involvement; (3) Do MRD results in BM and CSF correlate at specific time-points. Our data provide evidence that PCR-based detection of leukemic cells in CSF is indeed possible and is specific for CNS involvement.
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**MATERIALS AND METHODS**

**Patients and Materials**

In this retrospective analysis, bone marrow (BM) and cerebrospinal fluid (CSF) samples from 15 children with ALL, treated from 1983 to 1997 in the Emma Kinderziekenhuis AMC, were obtained at diagnosis and at several time points during the course of their disease. In each child BM and CSF samples were taken at least every 4 months during a period of 4 years after diagnosis. Five patients with a CNS relapse were included on basis of the availability of frozen CSF and BM samples at minimal five time-points during the two years the patient had been treated. Additionally ten control patients were selected, who were included on the base of absence of CNS relapse during long follow-up (95 months). Morphologic, immunophenotypic and cytogenetic analysis had been routinely performed at diagnosis and at relapse by standard techniques.

All patients were treated according to protocols of the Dutch Childhood Leukemia Study Group (DCLSG).\(^{13}\) Standard treatment of childhood ALL consists at least of a remission-induction course and central nervous system (CNS) prophylaxis, followed by maintenance therapy for two years. Depending on the protocol, a consolidation course was included. Intensified treatment was given to children identified as high-risk patients. For CNS prophylaxis only high dose methotrexate was used, in combination with intrathecal administration of methotrexate, dexamethason (DEX) and cytosine- arabinoside (ARA-C).

Diagnosis of CNS relapse was based on conventional cytology and flow cytometry. CNS leukemia was diagnosed if more than 5 cells per mm\(^3\) (or more than 15/3 cells) and on cytomorphologic examination lymphoblasts were seen in the CSF. Additionally, flow cytometric analysis of these CSF samples was performed. As standard monoclonal antibodies, CD45/CD14, CD3/CD20 and CD10/CD19 were used in flow cytometry. Additionally, the CD10/CD20 antibodies were used depending on the original immunophenotyping. Also cytospin preparations were made, stained with TdT and examined using a conventional microscope. In case of CNS or BM relapse, an additional reinduction treatment was given (consisting of vincristine, L-aparaginase, ARA-C and DEX).\(^{13}\)

**DNA isolation**

DNA was extracted from cryopreserved mononuclear cell fractions, isolated by Ficoll - Hypaque density gradient centrifugation, by means of the QIAamp Blood Kit (Qiagen, Dusseldorf, Germany) as described previously.\(^{48}\) For Southern blot analysis, DNA was digested with *BglII* (LifeTechnologies BRL, Gaithersburg, MD) and hybridized with the H24 JH probe\(^{116}\) and a J\(_{\delta}1\) probe.\(^{49}\) Incidentally, DNA of CSF and BM was obtained after
**PCR-based detection of CNS relapse in children with B-precursor ALL**

Table 1. Characteristics of patients

<table>
<thead>
<tr>
<th>Pt group</th>
<th>Pt</th>
<th>Age at diagnosis (years)</th>
<th>Time of 1st relapse from diagnosis (months) [site]</th>
<th>Karyotype</th>
<th>Immunotype</th>
<th>WBC.10⁶ cells at diagnosis</th>
<th>Outcome*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 1</td>
<td>1 1</td>
<td>13</td>
<td>38 [CNS]</td>
<td>46,XY t(7;9)</td>
<td>cALL</td>
<td>21</td>
<td>CCR</td>
</tr>
<tr>
<td>2 1</td>
<td>1 1</td>
<td>10</td>
<td>21 [CNS]</td>
<td>46,XY del13</td>
<td>cALL</td>
<td>7</td>
<td>† (64)</td>
</tr>
<tr>
<td>3 1</td>
<td>3 1</td>
<td>3</td>
<td>34 [CNS - BM]</td>
<td>46,XX</td>
<td>cALL</td>
<td>27</td>
<td>† (35)</td>
</tr>
<tr>
<td>4 1</td>
<td>2 1</td>
<td>3</td>
<td>34 [CNS - BM]</td>
<td>46,XY del11</td>
<td>cALL</td>
<td>39</td>
<td>CCR</td>
</tr>
<tr>
<td>5 1</td>
<td>1 1</td>
<td>12</td>
<td>34 [CNS - BM]</td>
<td>46,XY</td>
<td>cALL</td>
<td>14</td>
<td>† (117)</td>
</tr>
<tr>
<td>6 2</td>
<td>2 1</td>
<td>3.5</td>
<td>12 [BM]</td>
<td>&gt;50</td>
<td>cALL</td>
<td>69</td>
<td>† (50)</td>
</tr>
<tr>
<td>7 2</td>
<td>2 1</td>
<td>4.5</td>
<td>28 [BM]</td>
<td>-</td>
<td>cALL</td>
<td>145</td>
<td>CCR</td>
</tr>
<tr>
<td>8 2</td>
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<td>42 [testicular]</td>
<td>46,XY</td>
<td>cALL</td>
<td>171</td>
<td>CCR</td>
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</tr>
<tr>
<td>9 2</td>
<td>3 1</td>
<td>29 [BM]</td>
<td>46,XX</td>
<td>cALL</td>
<td>6</td>
<td>CCR</td>
<td></td>
</tr>
<tr>
<td>10 2</td>
<td>1 1</td>
<td>10</td>
<td>15 [BM]</td>
<td>46,XY</td>
<td>cALL</td>
<td>6</td>
<td>CCR</td>
</tr>
<tr>
<td>11 3</td>
<td>4 1</td>
<td>4</td>
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<td>cALL</td>
<td>32</td>
<td>CCR</td>
<td></td>
</tr>
<tr>
<td>12 3</td>
<td>4.5</td>
<td>-</td>
<td>46,XX</td>
<td>cALL</td>
<td>21</td>
<td>CCR</td>
<td></td>
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<td>222</td>
<td>CCR</td>
<td></td>
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<tr>
<td>14 3</td>
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<td>47,XY,+21</td>
<td>cALL</td>
<td>8.7</td>
<td>CCR</td>
<td></td>
</tr>
<tr>
<td>15 3</td>
<td>4 1</td>
<td>-</td>
<td>46,XX</td>
<td>cALL</td>
<td>33</td>
<td>CCR</td>
<td></td>
</tr>
</tbody>
</table>

**cALL:** common ALL; **CCR:** continuous complete remission

* Follow-up: 95 months; date of decease (months after diagnosis) is indicated in (..).

scraping from smears of BM and CSF (6 BM and 15 CSF slides from 15 patients). Erythrocytes were lysed for 5 minutes in 500 µl of water. Cells were centrifuged at 13,000g for 10 minutes. Pellets were resuspended in 500 µl of 0.1% w/v NP40 for 10 minutes at room temperature. Nuclei were pelleted by centrifugation at 13,000g for 10 minutes. Finally, proteinase K digestion was performed at 50°C for two hours, followed by 95°C for 10 minutes. DNA isolation was verified by amplification of the FcγRIIIb gene.52

Target identification on bone marrow samples taken at diagnosis and MRD analysis

All IGH and TCRD (V62/D63 or D82/D83) gene rearrangements were identified as described previously.345 All targets were checked for oligoclonality by Southern Blot analysis or heteroduplex analysis. For detection of MRD a two-round heminested allele-specific PCR was performed. In the first round, IGH and TCRD junctional regions were amplified with consensus primers FR3 and JH21; V62-3, D82-3 or D83-3 respectively.3 In the second round, a PCR was performed with a primer specific for the junctional region of one allele, the so-called allele-specific oligonucleotide (ASO) primer, in combination with the consensus primer JH21 or D83-3, respectively.3 Reactions were performed in mixtures as previously described. The input of genomic DNA in the first PCR was 1µg per reaction. The PCR was performed in a thermal cycler (Perkin Elmer Cetus Model 9600; Norwalk, CT) and consisted of 10 min at 95°C followed by 35 cycles of 30 sec at 95°C, 45 sec at
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60°C and finally 5 min at 72°C (total reaction volume 50 l). The second round of the heminested PCR had an input of 2 µl of PCR product, generated in the first reaction.

For each PCR, positive and negative controls were included. To avoid false-positive results, we compared the size of the PCR products of CSF samples with those of the PCR products from the BM sample positive at diagnosis. Specificity of amplification was tested on 1µg of DNA extracted from peripheral blood mononuclear cells from 10 different healthy donors. Water was used as a negative control.

To exclude false-positive results due to contamination with blood, results were evaluated from PCR of CSF samples from 5 patients without CNS relapse, taken at the moment the BM sample was positive. In none of the tested patients with BM relapse, the CSF samples were positive.

RESULTS

Fifteen patients with B-precursor ALL were studied, divided into three different groups: (1) patients that experienced a CNS relapse, in three cases accompanied by clinical BM relapse (n=5), (2) patients that had an isolated BM or testicular relapse without infiltration in the CSF (n=5) and (3) patients who remained in continuous complete remission (n=5). Characteristics of the patients are shown in Table 1. In all patients, analysis of MRD by PCR was performed in BM and CSF samples during follow-up of the disease (total number of 230 BM and CSF samples).

Description of five patients with CNS relapse (Figure)

Five patients (#1-5) experienced a CNS relapse. In one patient (#1) an isolated CNS relapse was diagnosed, in two patients (#2,3) CNS relapse was followed by a BM relapse (eight months after the first CNS relapse, in both patients), in one patient (#4) a combined CNS and BM relapse occurred and in one patient (#5) a BM relapse was noted 24 months before CNS relapse. In none of these patients, leukemic cells were detectable in the CSF at initial diagnosis by cytomorphology, immunology or PCR. In patient #1 and #2 immunophenotyping was not performed during follow-up. From patients #3, 4 and 5 immunophenotypic results were available. In four out of five cases with CNS relapse (#1,2,3,5), no residual leukemic cells (as analysed by PCR) in the bone marrow were present at the moment of CNS relapse.

In three of the patients (#1,2,3), leukemic cells were detected in the CSF by PCR four weeks before the diagnosis of CNS relapse was made by cytomorphologic techniques. In contrast, cytomorphologic examination (performed on all samples) and flow cytometric analysis (performed in #3 and #4) of these PCR-positive CSF samples did not show
Figure. MRD detection by PCR on BM and CSF samples in patients with CNS relapse

CNS: CNS relapse; BM: BM relapse; BMT: bone marrow transplantation
Start and cessation of treatment is marked under the graph of each patient. ■ BM morphology positive/PCR positive; □ BM morphology negative/PCR positive; □ BM morphology negative/PCR negative. Immunophenotyping results were only available for patient #3, 4 and 5. In the CSF samples that were PCR-positive and morphology-negative (■), the results of immunophenotyping were also negative.

evidence for the presence of blasts. Patient #4 showed evidence of molecular disease in the CSF and BM already 6 months before diagnosis of combined CNS and BM relapse.

In three cases (#2, 3 and 5), there appeared to be a persistently high level of molecularly detectable MRD in CSF at the time-point after relapse, although clinical
relapse was not yet diagnosed. The CSF sample in patient #5 was already PCR-positive/cytomorphology-positive during the third BM relapse, without clinical evidence of CNS relapse. The diagnosis of second CNS relapse was made two years after the third BM relapse (97 months after diagnosis). The persisting PCR-positivity of CSF after the third CNS relapse reflects the chemoresistance of this patient to the therapy. Due to the recurrent relapses, this patient was almost continuously treated until autologous bone-marrow transplantation (96 months). After BMT, a CNS and BM/CNS relapse occurred again and the patient died 115 months after initial diagnosis.

In patient #3 residual cells were repeatedly shown in the CSF by PCR analysis (time-points 5, 6 and 23 months). Also on BM analysis early PCR-positivity was noted (time-points 6 and 24 months). Cytomorphology and immunophenotyping of the CSF remained negative at those time points. Despite the detection of leukemic blasts by morphologic, immunophenotypic and PCR analysis at 24 months, this was clinically not considered as a CNS relapse due to absence of pleiocytosis. During treatment (until the 30th month after diagnosis) leukemic cells remained detectable in CSF with all techniques, but the first CNS relapse was ascertained by standard techniques after treatment was stopped (32 months after diagnosis).

In patient #2 it was interesting that the CSF sample, taken one month before isolated BM relapse, was positive in PCR analysis, whereas the BM sample remained negative at that time-point. At diagnosis of BM relapse, no leukemic cells were detectable in the CSF by PCR. All positive CSF samples from this patient were detected during treatment. This therapy included an extra induction course, which started after diagnosis of the first CNS relapse. There was a short treatment-free interval of 5 months. Patients #1 and #4 were off therapy at the moment of CNS relapse.

Description of 10 control patients

The 10 control patients consisted of five patients (#11-15) remaining in CCR without relapse after a follow-up period of 95 months and five relapsed patients (#6-10) without CNS involvement. Four relapses were localised in the BM, and one patient (#8) had an isolated testicular relapse. Every three months a CSF sample was examined during the first two years after initial diagnosis, followed by every six months during the subsequent two years (total of 60 CSF samples of 5 patients). In none of these specimens did PCR analysis, immunophenotyping or morphological examination reveal any leukemic cells, also at the moment of BM/testicular relapse. In all five relapsed patients, PCR results were positive in the BM at the time of BM relapse. This includes also the BM sample of patient # 8 at the moment of the isolated testicular relapse.
DISCUSSION

PCR-based monitoring of MRD allows detection of small numbers of malignant cells, not detectable with conventional techniques. Previous studies have shown that the response to therapy as measured by the presence of MRD in the BM at the end of induction therapy is an independent predictor of outcome in childhood ALL.\textsuperscript{3-5,7,9,11} Since discrimination of malignant cells and normal cells is often hard to make in CSF, the diagnosis of CNS involvement can only be made if an increased cell count in the CSF is noted. In theory, the detection of leukemic cells by PCR should also be possible in the CSF, in the absence of increased cell numbers, because the high sensitivity of the PCR allows detection of a single leukemic cell. This may enable earlier diagnosis and treatment of CNS relapse in comparison with other detection techniques.

In this study, we report on the application of PCR to perform molecular monitoring of MRD in the CSF. Three different groups of patients were compared: (1) five patients experiencing seven CNS relapses, in three cases accompanied by clinical BM relapse, (2) five patients developing an isolated BM (\textit{n}=4) or testis relapse (\textit{n}=1) without infiltration in the CNS and (3) five patients remaining in continuous complete remission. In four out of four isolated CNS relapses, the CNS relapse was not accompanied by the presence of PCR-detectable residual cells in the bone marrow. These results indicate that PCR analysis of CSF can be used to distinguish between isolated CNS disease and combined CNS/BM relapse in children with B-precursor ALL, which is in agreement with previous studies.\textsuperscript{114,116,117} Contrasting results were obtained for T-ALL; Neale \textit{et al.}\textsuperscript{115} showed in four out of six patients the concomitant presence of MRD in BM during CNS relapse. Conversely, all five patients in our series that had relapsed in BM or testis were negative for residual disease in the CSF at the time of relapse as well as in follow-up samples. Leukemic cells were also undetectable by PCR in any of the CSF samples of the five patients remaining in CCR (follow-up \textit{>95} months). This absence of false-positive PCR results in CSF samples of the described patients underlines the specificity of the PCR approach. It indicates that PCR is indeed suitable for analysis of MRD in the CSF.

PCR-positive samples were only found in patients that had already experienced a CNS relapse before or in patients that suffered from a CNS relapse later in their disease. PCR-positivity preceded a CNS relapse in five of the six episodes in which CSF was tested by PCR, prior to diagnosis of CNS relapse by standard techniques. Cytomorphologic analysis was negative for leukemic blasts in these CSF samples. Also immunophenotyping did not influence the time point at which a relapse was diagnosed by cytomorphological analysis, although these results were not available in all patients. In one case (CNS1 in patient #5) no CSF sample prior to diagnosis of relapse was tested. In the only PCR-negative case, the last CSF sample was tested 12 months prior to diagnosis of CNS relapse, whereas in three of
the PCR-positive cases the first PCR-positive CSF sample was only one month prior to CNS relapse (CNS1 in patient #1, CNS1 and BM1 in patient #2, and CNS1 in patient #3). These results show that molecular detection of leukemic cells in the CSF detects CNS relapse at an earlier moment than with conventional techniques. As we have shown that in this small patient group every positive CSF sample at any time inevitably leads to CNS relapse, the question arises whether treatment should be modified on the base of PCR analysis of CSF. Early intensification of CNS treatment may help prevent CNS relapse and boost survival rates. The absence of PCR-detectable leukemic cells in CSF at primary diagnosis suggests that PCR analysis of CSF at diagnosis can not be used to determine an increased risk for CNS relapse. This is in contrast with the study by Hooijkaas et al., who reported that all CNS relapses occurred in patients with TdT-positive cells in the CSF at diagnosis.

In three out of five patients (#2, 3 and 5), malignant cells were found for a prolonged period after starting therapy. All patients were still under chemotherapeutic treatment at these time points. Moreover, all these patients relapsed several times and did not survive in the end. Probably, the persistent presence of leukemic cells reflects that maintenance therapy causes a smouldering of CNS leukemia.

A theoretical drawback of the PCR approach on CSF samples is the risk of false-positive results – due to contamination with blood. This contamination is possible in the procedure of a lumbar puncture and is sometimes even macroscopically seen. Results from PCR of CSF samples from patients without CNS relapse taken at the moment that the BM sample was PCR positive, showed no positivity, which indicates that false-positive results caused by (traumatic) blooded mixture did not occur frequently. Probably, the level of contamination is too low to result in false-positive results.

We conclude that PCR-based studies provide, in conjunction with morphological and immunocytochemical methods, an accurate diagnosis of CNS leukemias. Although flow cytometry is nowadays performed in a routine setting as a reliable method and has shown to be easy and quick to perform, our results demonstrate that monitoring of CSF by PCR gives additional possibilities for early detection of a CNS relapse. Since our results are retrospectively obtained from a small group of patients and rigidly blinded prospective analysis was not performed, it is still difficult to incorporate PCR of CSF into clinical practice, based on these results only. The PCR technique is interesting but requires further testing. Hence, PCR of CSF may be a useful adjunctive tool in diagnosis of CNS relapse and may be recommended as an additional detection technique, besides the conventional cytology and flow cytometry, on which clinical decisions can be made. Since MRD analysis is nowadays routinely performed in BM and peripheral blood, especially since recently the simplified real-time quantitative PCR (RQ-PCR) with TaqMan probes
has become available, detection of malignant cells in CSF samples seems feasible. Most of the laboratory preparations, i.e. development of the patient-specific marker, have already been performed in each case. Therefore, PCR analysis of CSF can easily be implemented in the diagnostic approach. Subsequently, in a larger prospective clinical study on larger numbers of patients, the prognostic value of PCR analysis in comparison with the standard detection techniques can be evaluated.