Interphase fluorescent in situ hybridization deletion analysis of the 9p21 region and prognosis in childhood acute lymphoblastic leukaemia (ALL): results from a prospective analysis of 519 Nordic patients treated according to the NOPHO-ALL 2000 protocol

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Summary

Interphase fluorescent in situ hybridization (FISH) was applied on diagnostic BM smears from 519 children with acute lymphoblastic leukaemia (ALL) in order to establish the frequency and prognostic importance of 9p21 deletion in children enrolled in the Nordic Society of Paediatric Haematology and Oncology (NOPHO) – 2000 treatment protocol. Among the patients, 452 were diagnosed with B-cell precursor (BCP)-ALL and 66 with T-ALL. A higher incidence of 9p21 deletions was found in T-ALL (38%) compared to BCP-ALL (15-87%). Homozygous deletions were found in 19-7% of T-ALL and 4-0% of BCP-ALL; hemizygous deletions were found in 18-2% and 11-7% respectively. In our series, 9p21 deletions were detected in all age groups with a steady rise in the frequency with age. There was no significant difference in outcome between cases with or without 9p21 deletion or between cases with hemi- or homozygous deletions of 9p21. In conclusion, in this large series of childhood ALL deletion of 9p21 was not associated with worse prognosis. However, interphase FISH deletion analysis of 9p21 could be used as a first step to detect unfavourable subtle cytogenetic aberrations such as the dic(9;20) rearrangement.

Keywords: childhood acute lymphoblastic leukaemia, 9p21 deletion, interphase fluorescent in situ hybridization.
T-ALL and one-third of BCP-ALL cases) (Bertin et al, 2003; Takeuchi et al, 1997). In patients with T-cell ALL, homozygous deletion of 9p21 has been associated with an adverse prognosis, but the significance of this event in BCP-ALL remains controversial and an independent prognostic significance has been difficult to establish (Calero Moreno et al, 2002; Mirebeau et al, 2006; Moorman et al, 2010; van Zutven et al, 2005; Yamada et al, 1997).

The 9p21 region contains three genes that were found to be involved in the development of different tumours: CDKN2A, CDKN2B and MTAP. CDKN2A is a tumour suppressor gene that encodes two proteins, and acts through the Rb and MDM2 pathways. CDKN2B is a tumour suppressor gene, the action of which through the Rb pathway is complementary to CDKN2A (p16) (Chim & Kwong, 2006). MTAP is located approximately 100 kb telomeric to CDKN2A and encodes methylthioadenosine phosphorylase, an enzyme involved in purine and methionine metabolism. Loss of MTAP has been suggested to make cancer cells more sensitive to drugs that interfere with folate metabolism (Batova et al, 1996). Deletions of 9p21 vary in size and may cover large genomic regions, spanning between 0·1 and >30 Mb (Florl & Schulz, 2003). Therefore, the MTAP gene, which is located approximately 100 kb telomeric to CDKN2A, is often co-deleted in ALL.

Investigation of the 9p21 deletion frequency and its prognostic significance in ALL has been hampered by the absence of simple and readily available diagnostic methods. Most of the earlier analyses were based on Southern blotting, which is reliable but laborious and requires large amounts of material, which is not always available from ALL patients (Calero Moreno et al, 2002; Heyman & Einhorn, 1996). Therefore, some of these studies may have been biased by the preferential selection of samples from patients with high tumour burden and high white blood cell (WBC) count, usually referred to a high risk group leading to under-representation of standard risk samples with low leucocyte counts. Today, the use of high-resolution genomic arrays, as well as quantitative polymerase chain reaction (PCR) analysis gives new opportunities to identify smaller deletions, but these methods require saved DNA. Interphase fluorescent in situ hybridization (FISH) provides easy and quick detection of translocations and deletions on bone marrow (BM) smears from unselected ALL patients. Other advantages of this method are the availability of smears for FISH analysis from diagnostic BM samples and small demands on their storage. Interphase FISH is robust and easy to perform, and thus an attractive diagnostic tool.

The aim of our study was to investigate a large cohort of children with ALL for the presence of 9p21 deletion using interphase FISH, in order to establish the frequency of 9p21 deletion in a Nordic population, and to evaluate its importance as a prognostic marker, as well as to clarify the detection sensitivity of the FISH probe and determine the importance of its use as a diagnostic tool in childhood ALL.

Materials and methods

Patients

All participating centres of the Nordic Society of Paediatric Haematology and Oncology (NOPHO) were invited to take part in the study with the aim of investigating the frequency and prognostic importance of 9p21 deletion in children with ALL. The treatment protocol NOPHO-2000 was started in 2001 and provided a unique opportunity to assess the prognostic importance of 9p21 deletion in a large cohort of patients diagnosed and stratified using uniform criteria and treated with the same protocol. Between 2001 and 2006, 1173 children (<18 years) were diagnosed with ALL and treated in paediatric departments in the Nordic countries (Denmark, Finland, Iceland, Norway and Sweden). Of these, 885 were included in the NOPHO ALL-2000 protocol. Altogether, diagnostic BM smears from 626 patients were analysed with interphase FISH, constituting 53·4% of all paediatric patients diagnosed with ALL 2002–2006 and 519 (58·6%) of all patients included in the NOPHO ALL-2000 protocol during this time. The final analysis was performed on 519 patients who met the study criteria.

Risk-stratification and therapy

The stratification and therapy in the NOPHO ALL-2000 protocol has been described in detail elsewhere (Schmiegelow et al, 2010). An overview of the stratification and therapy plan for the different treatment-arms can be seen in Fig S1. The patients were stratified into patients with lower risk (LR) features at diagnosis [standard (SI) and intermediate intensity (II) therapy groups] and patients with higher risk (HR) features [Intensive (Int), Very Intensive (VI) and Extra Intensive (EI) therapy groups]. Patients were LR if they had: B-precursor phenotype with a WBC count £50 × 10⁹/l, no central nervous system (CNS)/testicular involvement, no adverse cytogenetic changes (Ph+, MLL-rearrangement, t(1;19), hypodiploidy <45 chromosomes), good response to induction therapy (≤25% blasts in BM: M1/M2 at day 15 and <5% blasts in BM: M1 at day 29). Patients aged 1–9·99 years at diagnosis and WBC count ≤100 × 10⁹/l were stratified into the SI group. The remaining patients in the LR-group were stratified into the II group. The HR group had one or more of the high-risk features. Patients ≥5 years at diagnosis with WBC count 100–200 × 10⁹/l, T-cell, mediastinal mass or CNS-involvement were stratified into the VI group and given cranial irradiation as a part of the CNS-directed therapy. Patients with at least one of: WBC count >200 × 10⁹/l, Ph+, very poor response (M3 BM day 29), hypodiploidy <34 chromosomes, age >10 years and MLL-rearrangement were stratified into the EI group, which aimed for allogeneic stem-cell transplantation (SCT) in first complete remission (CR1). All other HR-patients were stratified into the Int therapy group.
Statistical analysis

The Statistical Package for the Social Sciences (spss) software for Windows 17.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses. The probability of event-free survival (pEFS) was calculated using the Kaplan–Meier method and the different cytogenetic and clinical subgroups were compared using the log rank test. The significance limit for P-values was set to 0.05 in all tests. The Chi-square test with exact calculation of P-value was used to investigate possible correlations between cytogenetic groups and clinical characteristics. Multivariate analyses using Cox regression models were performed to identify cytogenetic and clinical factors with independent impact on EFS and interactions between these factors. In the analysis of EFS, events comprised induction failure, relapse, and failures, death in remission, relapse and second malignancy. In the overall survival analysis, death was the only endpoint. The median observation time for surviving patients in the study was 53 months (12–96 months). The date of last follow-up was 26 January, 2009.

FISH-analysis

All samples were analysed with the LSI p16 (9p21) FISH probe (Abbot Molecular Inc., IL, USA). The probe represents a mixture of the CDKN2A (p16) probe labelled with Spectrum Orange and a CEP9 probe, labelled with Spectrum Green. The p16 probe spans approximately 190 kb and covers the 9p21 region, including D9S1749, CDKN2A, CDKN2B, c9orf53, MTAP and D9S1752. Slides were pretreated, labelled, hybridized and washed according to the manufacturers’ instructions. DAPI (4’,6-diamidino-2-phenylindole) was applied to ensure visualization of nuclei and analysis was performed under a fluorescent microscope. The signals were visualized using a Zeiss Axioplan fluorescence microscope equipped with a cooled charge-coupled device-camera, controlled by a Macintosh power computer. Grey scale images were captured, pseudocoloured and merged using the SmartCapture software (Digital Scientific, Cambridge, UK). When possible, 200 nuclei were analysed per sample.

Results

Clinical characteristics and other prognostic parameters of the 519 patients included in the study were compared with the parameters of the patients treated according to the NOPHO ALL-2000 protocol, but whose BM smears were not provided for analysis and revealed no significant bias or selection of patients apart from T-cell cases, which were slightly more likely to be analysed (P = 0.047, Table I). The reason for this is unclear, but may be due to a higher motivation to submit slides for T-cell cases because of previous findings or may be a chance finding considering possible correction for multiple comparisons. In the BCP-ALLs that were included in our study, prognostically important cytogenetic abnormalities followed the usual distribution in Nordic patients; high hyperdiploidy in 157 patients (34%), ETV6/RUNXI rearrangement in 102 patients (22%), MLL rearrangement in nine patients (2%), PBX1/TCF3 in 18 patients (4%), hypodiploidy in 7 (1.5%), BCR/ABL1 in two patients (0.4%) and intrachromosomal amplification of 21q in nine patients (2%). The low incidence of Ph+ ALL was due to the introduction of the EsPhALL (European Intergroup Study on Post Induction Treatment of Philadelphia Positive Acute Lymphoblastic Leukaemia with Imatinib) -protocol, in the latter part of the protocol era.

T-ALL

Sixty-six of the 519 patients had T-ALL and deletion of 9p21 was found in 25 patients (37%), which was homozygous in 11 (16%), hemizygous in 12 (18%) and homo/hemizygous in two (3%). The majority of the T-cell patients with 9p
deletion did not have the karyotypic changes that are common in BCP-ALL (Table SI). In this group, there was one Ph+ case, two patients with MLL rearrangement and three patients with high hyperdiploidy. The 9p21 status and main clinical parameters are shown in Table SII. Kaplan–Meier survival analysis indicated that the patients with hemizygous loss of 9p21 fared slightly better than those with homozygous deletion and no deletion \[pEFS \text{ at 5 years} \pm \text{standard error (SE)} = 0.756 \pm 0.107, 0.61 \pm 0.14, 0.63 \pm 0.08, \text{respectively}\], but the difference did not reach statistical significance \(P = 0.38\) (Fig 1). In this analysis, patients with homo- or hemizygous deletion were grouped together with homozgyously deleted patients. Of the two patients with both homo- and hemizygous deletions one did not relapse and the other had induction failure. The result of this analysis did not change if the patients with hemizygous deletion were grouped together with patients with no deletion, identifying the homozgyously deleted group as the only one with certain gene inactivation.

**BCP-ALL**

A total of 452 patients fulfilled the inclusion criteria for the NOPHO ALL-2000 protocol. Deletions of 9p21 were observed in 71 patients (15.7%): homozygous in 17 (3.8%), hemizygous in 53 (11.7%), homo-/hemizygous in 1 (0.2%), the latter was treated as homozgyously deleted in the survival analyses (Fig 2). The distribution of 9p deletions according to prognostically important genetic aberrations is shown in Table II.

Kaplan–Meier survival analysis demonstrated a significant difference in the pEFS of patients with and without deletion of 9p21 \(P = 0.04\) for the overall comparison and \(P = 0.14\) when patients with deletions were grouped together. The 5-year EFS of patients with hemizygous and homozygous deletion was the same and slightly lower than that of patients with no deletion \(pEFS\) for patients with homozygous deletion \(\pm SE\) was \(0.756 \pm 0.107\), for the group with homozgyous deletion \(0.757 \pm 0.065\), and for patients with no deletion \(0.829 \pm 0.02\).

9p21 deletion in different risk groups

Out of 452 patients with BCP-ALL who were included into the survival analysis, 333 children were assigned to the LR-group (SI and II-patients) in the NOPHO-2000 protocol and 119 children were assigned to the HR-group (Int, VI and EI-patients). There was a significantly higher fraction of patients with any type of the 9p21 deletion in the high risk group compared with the low risk group, 10.9% and 29.4%, respectively \(P = 0.0001\). Kaplan–Meier survival analysis was performed on patients with low and high risk of relapse separately, but did not show any significant difference in EFS survival in patients with and without deletion of 9p21 in either risk categories. Patients in the low risk group with homozygous or hemizygous deletion had lower EFS-value \(pEFS \pm SE = 0.76 \pm 0.148\) and \(0.78 \pm 0.088\), respectively), while patients without deletion had higher EFS \(pEFS \pm SE = 0.83 \pm 0.045\). This was in contrast to the high-risk group, in which the patients with homozygous and hemizygous deletion had better outcome \(pEFS \pm SE = 0.75 \pm 0.153\) and \(0.74 \pm 0.094\), respectively), whereas patients with no deletion fared less well with an EFS of \(0.68 \pm 0.053\).

We also investigated whether deletion of 9p21 may serve as a prognostic marker for delayed relapses in patients with BCP-ALL. We selected patients who were off primary therapy at the time of last follow-up and calculated a time from cessation of therapy according to the therapy-protocol, excluding patients who were treated with SCT in CR1. In our cohort 345 patients fulfilled these criteria and 51 had 9p21 deletion. Survival analysis demonstrated earlier failures in patients with homozygous and hemizygous deletions after cessation of therapy compared to the outcome of patients with no deletion, but similar end results \(P = 0.265\) (Fig 3A). When stratification of patients according to risk group was performed we found that the early separation of the curves in the overall analysis was
due to an effect in the high-risk patients (Fig 3B), whereas no
difference was detected for patients with low risk criteria.
However, this difference did not reach statistical significance
\( (P = 0.11) \). Cox regression did not reveal any relevant predic-
tors or adverse outcome after cessation of therapy.

**Analysis of cytogenetic subgroups**

No significant difference in EFS of patients with or without
deletion of 9p21 was observed neither in the whole cohort of
patients bearing high hyperdiploidy or \( ETV6/RUNXI \)
rearrangement nor in patients with follow-up longer than 2 years.
The low number of events in these patient groups makes the
results difficult to interpret and may need longer follow-up
and additional patients to be meaningful. Analysis of EFS in
other cytogenetic subgroups, \( TCF3/PBX1 \) rearrangement, and
‘normal’ karyotype did not demonstrate any significant
difference in the survival of patients with and without deletion
of 9p21. It is worth mentioning that patients with dic(9;20)
had either type of 9p21 loss, but hemizygous deletion was the
most frequent.

**Discussion**

In this large prospective study we evaluated the frequency of
interphase FISH-detectable 9p21 deletions in Nordic children
with ALL. The unique feature of our work is that it comprises
the analysis of one of the largest unselected collections of
consecutively diagnosed patients who were treated according
to a uniform protocol (519 cases), which strengthens the
power of the analysis. About 60% of all patients diagnosed in
the Nordic countries between 2002 and 2006 were included in
the study and we could show that there was no bias towards
unfavourable prognostic factors, which has hindered some of
the earlier studies (Calero Moreno et al, 2002).

Interphase FISH has proved to be a fast and reliable method,
although it has one apparent drawback – the large size of the
probe (200–300 kb), which may lead to misinterpretation of
the results in cases with smaller deletions that escape detection.
This has already been noted in a study of the frequency of 9p21
deletions in Ewing sarcoma (Savola et al, 2007). One can thus
discuss the impact of the co-deletion of all three tumour
suppressor genes in the region. Four large studies utilizing
interphase FISH with a commonly used commercial probe to
study 9p21 deletions in patients with ALL have been published
to date and the observed deletion frequency was 20–27% (Mullighan
et al, 2008; Perez-Vera et al, 2008; Sulong et al, 2008; Woo
et al, 2005).

Thus, the 20% frequency of deletion observed in our study is
in line with earlier reports. Nevertheless, the increasing use of
high-resolution genomic arrays, as well as quantitative PCR
analysis, indicate that the real prevalence of 9p21 loss is higher
and reaches 40–50% in children with ALL and points out that
deletions of this locus may be both small and large (Kawamata
The analysis performed in this study focused on the prognostic
significance of large deletions of 9p21 (>200 kb), which in
most cases are beyond the resolution of conventional
G-banding.

T-ALL and BCP-ALL are biologically different entities and
thus the analysis of patients with T-cell or BCP immuno-
phenotypes was largely performed separately. As expected, the
frequency of 9p21 deletion in T-ALL was higher than in BCP-
ALL. The overall frequency of 9p21 deletion of 38% in patients
with T-ALL was lower than in earlier reports (Bertin et al,
2003; Ramakers-van Woerden et al, 2001), and frequencies of
hemizygous and homozygous deletions were surprisingly
equal. To the best of our knowledge, a few studies that
investigated an association between hemizygous loss of 9p21
and prognosis of ALL in children (Carter et al, 2001; Kees et al,
had limited amounts of patient samples and thus could
not analyse patients with BCP-ALL and T-ALL separately.
Our analysis revealed no difference in the survival of
patients with homozygous deletions in comparison with the
rest of the patients with T-ALL. However, we observed that
patients with T-ALL and hemizygous deletion of 9p21 had the
best survival, whereas the prognosis of patients with homo-
yzogous and no deletions of 9p21 was similar. Although these
findings did not reach statistical significance, the observation is
in line with an earlier study performed with Southern blotting
and single-strand conformation polymorphism (Diccianni
et al, 1997). We observed no change in deletion frequency in
different age groups, which is in line with a recently published
analysis of 266 patients with T-ALL (Sulong et al, 2008). The
results of our analysis suggest that T-ALL is a more homo-
genous disease in relation to the occurrence of 9p21 deletions
that is independent of age and other established risk factors.

A possible difference in survival of patients with hemizygous
and homozygous deletions of 9p21 may indicate involvement of
different pathways in development of T-ALL in patients
with hemizygous deletion of 9p21.

In contrast to T-ALL, analysis of patients with BCP-ALL
revealed a high variation of 9p21 deletion rates with age, WBC
count and risk groups.

The highest frequency of 9p21 deletions in patients with
BCP-ALL was detected in patients with dic(9;20) (100%), and
TCF3/PBX1 rearrangement (38%). Dic(9;20) is a subtle
abnormality that may easily be mistaken for monosomy 20
and/or del(9p) when using chromosome banding alone and
many cases are undetected unless FISH analysis is performed
(Forestier et al, 2008). Presence of 9p21 deletion may serve as
an indication of dic(9;20), especially in presence of additional
changes in the karyotype, e.g. monosomy 20. Any prognostic
significance of particularly hemizygous deletion of 9p21 may
be confounded by this association.

Previous reports have shown a very low frequency of 9p21
deletions in patients with TCF3/PBX1 rearrangement
(Maloney et al, 1998; van Zutven et al, 2005). However, in
our hands, interphase FISH demonstrated a high incidence of
9p21 deletions in this patient group. We have no obvious
explanation for this discrepancy, but these results are in
accordance with a recent report of a 40% deletion rate in 25
patients with this aberration (Sulong et al, 2008). The
higher prevalence of 9p21 deletions in patients with TCF3/PBX1
rearrangement may only partly be explained by a high
incidence of i(9q) that was diagnosed in two out of eight
patients with such an aberration in our material (Table SIII).
Thus it is the most common imbalance in this cytogenetic
subgroup, suggesting that inactivation on 9p21 locus is an
important step of the pathogenesis of ALL with the TCF3/
PBX1 fusion.

The role of hemizygous deletion of 9p21 in BCP-ALL has
not been thoroughly investigated previously. The resolution of
the majority of earlier studies precluded correct diagnosis of
hemizygous deletion and could thus not specify their prog-
nostic significance. In analysis of BCP-ALL, in contrast to the
situation in T-ALL, Kaplan–Meier survival analysis demon-
strated no difference in EFS of patients with and without
deletion of 9p21 (Fig 2).

Time to relapse is of great prognostic importance for
subsequent relapse therapy in ALL. Therefore, relapses are
frequently divided into early (occurring before cessation of the
therapy or immediately thereafter) and late (usually occurring
greater than at least 6 months after the end of therapy). Late
relapses predominantly occur in the lower risk-groups and in
the large childhood cytogenetic groups (high hyperdiploidy
and t(12;21)). However, there are few factors capable of
predicting them in high-risk cases. Analysis of patients with
discontinued therapy detected a trend towards a worse
outcome for high-risk patients with 9p-deletions compared
with patients without deletions; however this difference did
not reach statistical significance.
Despite the large number of patients in the low risk group, the low incidence of death as well as the low number of events in this group results in lack of power for this comparison and the issue of prognostic importance of 9p21 may never be satisfactorily resolved for this category of patients. The analysis of prognostic influence of 9p21 deletion in cytogenetic subgroups as well as the analysis of late relapses, occurring after cessation of treatment, was also performed separately in patients with high hyperdiploidy and ETV6/RUNX1 rearrangements. The paucity of relapses in these groups explains difficulties in finding factors predisposing for adverse prognosis in such patients with otherwise favourable prognosis. It is extremely difficult to single out in common factors in <6% of cases and establish their independent prognostic significance, since there may be different reasons for relapse. In our study, deletions of 9p21 had no negative impact on the prognosis in patients with high hyperdiploidy or ETV6/RUNX1 rearrangement. It has to be pointed out that homozygous or hemizygous deletion of 9p21 was detected only in eight patients with high hyperdiploidy in this large study. Thus all results should be treated with caution.

In conclusion, this large prospective study has investigated the frequency of large 9p21 deletions and their impact on the prognosis of T-ALL and BCP-ALL in a Nordic population. The overall frequency of 9p21 deletion in our study does not differ from that reported by other groups. We have observed high frequencies of 9p21 deletion in patients with T-cell ALL, TCF3/PBX1 rearrangements, as well as in cases with dic(9;20), and low frequencies in patients with high hyperdiploidy and ETV6/RUNX1 rearrangements. Even if no major impact of 9p21 deletion could be found, except perhaps for late relapses in the BCP high-risk group, it may still be important to diagnose deletions of 9p, as a characteristic of the dic(9;20), a genetic aberration which may otherwise be overlooked.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Schematic representation of risk stratification and treatment of patients with ALL in NOPHO-2000.
Table S1. Karyotypes of patients with T-ALL and deletion of 9p21 region.
Table SII. Distribution of main clinical parameters in patients with T-ALL and their correlation with 9p21 deletions pattern. (Homo-/hemizygous deletion grouped with homozygous deletions).
Table SIII. Karyotypes of patients with BCP-ALL and deletion of 9p21 region.

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