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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 (15·7%). 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.

Inactivation of the 9p21 region has been reported in numerous malignant tumours, e.g. melanoma, mesotheliona, urinary bladder cancer, chordoma, gastrointestinal tumours (Chiosea *et al*, 2008; Haller *et al*, 2008; Hallor *et al*, 2008; Walker *et al*, 1998), as well as in acute lymphoblastic leukaemia (ALL) (Heyman & Einhorn, 1996; Mullighan *et al*, 2007; Ogawa *et al*, 1995; Tsuzuki *et al*, 2007). The prognostic importance of 9p21 deletion in childhood ALL has been debated for a long time (Calero Moreno *et al*, 2002; Faderl *et al*, 1999; Graf Einsiedel *et al*, 2001; Mirebeau *et al*, 2006). It is well recognized that the frequency of homozygous deletion in T-ALL is higher than in B-cell precursor (BCP)-ALL (approximately two-thirds of

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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 $\leq 50 \times 10^9$/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 $\leq 10{\cdot}0 \times 10^9$/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 $100{\cdot}1–200 \times 10^9$/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 $>200 \times 10^9$/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 9p21 probe spans approximately 190 kb and covers the 9p21 region, including D9S1749, C*DKN2A*, *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 Axioplane 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 (34·7%), *ETV6/RUNX1* rearrangement in 102 patients (22·6%), *MLL* rearrangement in nine patients (2·0%), *PBX1/TCF3* in 18 patients (4·0%), hypodiploidy in 7 (1·5%), *BCR/ABL1* in two patients (0·4%) and intrachromosomal amplification of 21q in nine patients (2·0%). 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.

Table I. Clinical characteristics of the patients treated according to the NOPHO ALL-2000 protocol over the same time period, who were analysed or not by interphase FISH.

Parameter	Analysed with FISH	Not analysed with FISH	Total	*P*
Sex			883	0·348
Male^{R}	296	196	492	
Female	223	168	391	
Age, years			883	0·072
1–5^{R}	329	239	568	
6–9	106	54	160	
10–14	84	71	155	
WBC, 10^9/l			883	0·101
≤10^{R}	283	179	417	
10·1–50	158	123	281	
50·1–100	56	31	98	
≥100·1	67	31	98	
Immunophenotype			881	0·047
BCP-ALL	452	332	784	
T-ALL	66	31	97	
Risk-group			883	0·679
Standard	181	132	313	
Intermediate	153	118	271	
Intensive	101	66	167	
Very intensive	38	23	61	
Extra intensive	46	25	71	
Mediastinal mass			871	0·827
Yes	38	25	63	
No^{R}	476	332	808	
CNS leukaemia			880	0·677
Yes	13	11	24	
No^{R}	505	351	856	

WBC, white blood cell count; BCP-ALL, B-cell precursor acute lymphoblastic leukaemia; T-ALL, T cell acute lymphoblastic leukaemia; CNS, central nervous system.
R-used as reference group in Cox-regression analysis.

T-ALL

Sixty-six of the 519 patients had T-ALL and deletion of 9p21 was found in 25 patients (37·9%), which was homozygous in 11 (16·7%), hemizygous in 12 (18·2%) and homo/hemizygous in two (3%). The majority of the T-cell patients with 9p

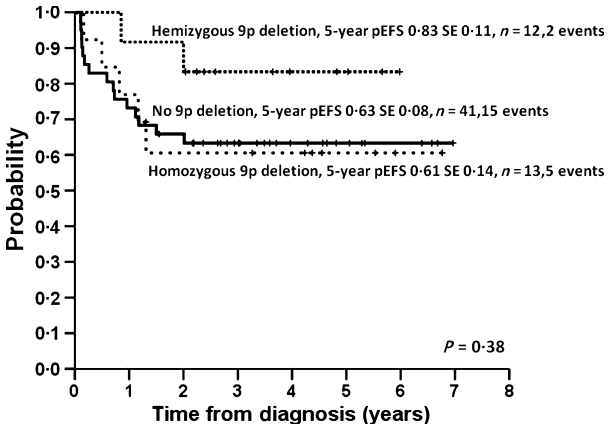

Fig 1. Kaplan–Meier survival analysis of 66 patients with T-ALL stratified by deletion status.

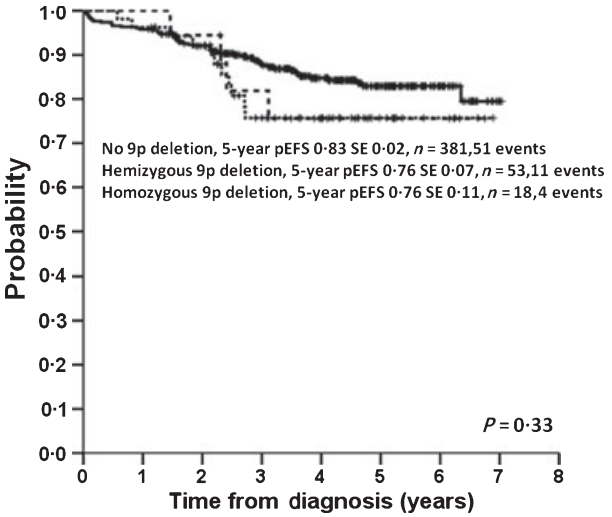

Fig 2. Kaplan–Meier survival analysis of 452 patients with BCP-ALL stratified by deletion status.

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 at 5 years ± standard error (SE) = 0·83 ± 0·11, 0·61 ± 0·14, 0·63 ± 0·08, respectively], but the difference did not reach statistical significance ($P = 0·38$) (Fig 1). In this analysis, patients with homo-/hemizygous deletion were grouped together with homozygously 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 homozygously 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 homozygously 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 no significant difference in the pEFS of patients with and without deletion of 9p21 ($P = 0·33$ 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 ± SE was 0·756 ± 0·107, for the group with hemizygous deletion 0·757 ± 0·065, and for patients with no deletion 0·829 ± 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 ± SE = 0·76 ± 0·148 and 0·78 ± 0·088, respectively), while patients without deletion had higher EFS (pEFS ± SE = 0·83 ± 0·045). This was in contrast to the high-risk group, in which the patients with homozygous and hemizygous deletion had better outcome (pEFS ± SE = 0·75 ± 0·153 and 0·74 ± 0·094, respectively), whereas patients with no deletion fared less well with an EFS of 0·68 ± 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

Table II. Distribution of 9p21 deletions among cytogenetic subgroups in BCP-ALL. Numbers in brackets = % of deleted cases within each group of aberration and for totals % of the whole population.

	9p21 deletion results				
Karyotype	Homozygous	Hemizygous*	Homo/hemizygous	No deletion	Total
MLL	0	0	0	9 (100%)	9 (2·0%)
BCR/ABL1	1 (50%)	1 (50%)	0 (0%)	0 (0%)/0	2 (0·4%)
Hypodiploid	3 (42·9%)	0 (0%)	0	4 (57·1%)	7 (1·5%)
TCF3/PBX1	0	8 (44·4%)	0	10 (55·6%)	18 (4·0%)
Hyperdiploid	3 (2·7%)	6 (4·4%)	1 (1·1)	147 (91·8%)/	157 (34·7)
ETV6/RUNX1^{R}	5 (29·4%)	9 (17·0%)	0 (0%)	88 (86·3%)	102 (22·6%)
amp21	1 (11·1%)	0 (0%)	0	8 (88·9%)	9 (2·0%)
Other	2 (3·1%)	8 (12·5%)	0	54 (84·4%)	64 (14·2%)
Normal	1 (2·0)	2 (4·1)	0 (0)	46 (93·9)	49 (10·8)
No result	1 (4·8%)	5 (23·8%)	0	15 (71·4%)	21 (4·6%)
Total	17 (3·8)	53 (11·7)	1 (0·2)	381 (84·3)	452 (100)

*Includes also patients with only one centromere 9, detected by FISH.
R-used as reference category in Cox-regression analysis.

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 predictors 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/RUNX1* 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 *et al*, 2008; Mullighan *et al*, 2007; Kustanovich *et al*, 2005). 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 immunophenotypes 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*,

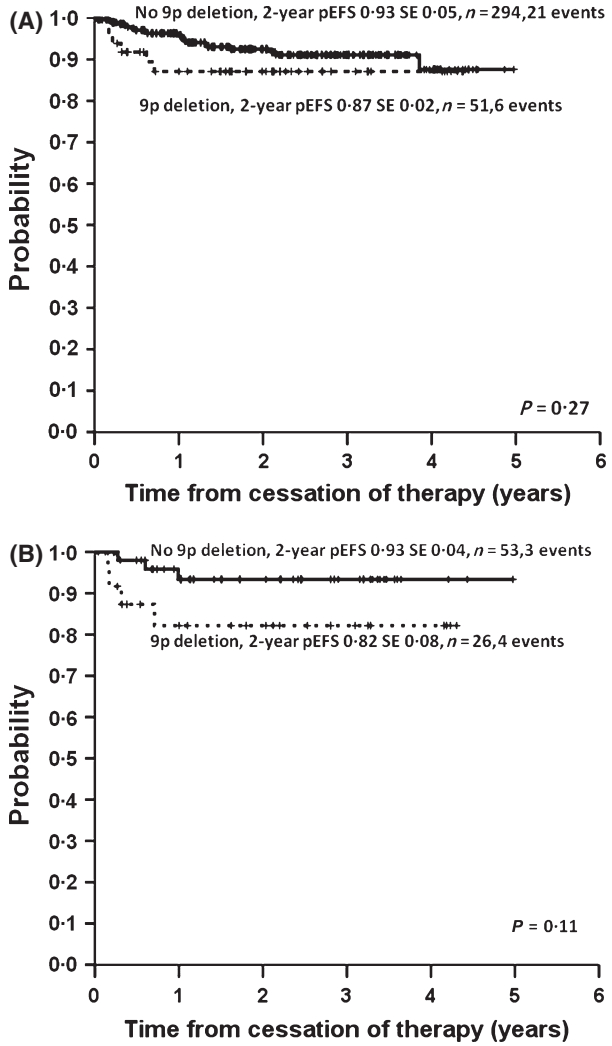

Fig 3. Kaplan–Meier survival analysis of 345 children with BCP-ALL followed after cessation of therapy. (A) The whole population, (B) Only high risk cases.

1997) 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 homozygous 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 homogeneous 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 prognostic significance. In analysis of BCP-ALL, in contrast to the situation in T-ALL, Kaplan–Meier survival analysis demonstrated 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 deletion 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 SI. 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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References

Batova, A., Diccianni, M.B., Nobori, T., Vu, T., Yu, J., Bridgeman, L. & Yu, A.L. (1996) Frequent deletion in the methylthioadenosine phosphorylase gene in T-cell acute lymphoblastic leukemia: strategies for enzyme-targeted therapy. *Blood*, **88,** 3083–3090.

Bertin, R., Acquaviva, C., Mirebeau, D., Guidal-Giroux, C., Vilmer, E. & Cave, H. (2003) CDKN2A, CDKN2B, and MTAP gene dosage permits precise characterization of mono- and bi-allelic 9p21 deletions in childhood acute lymphoblastic leukemia. *Genes Chromosomes Cancer*, **37,** 44–57.

Calero Moreno, T.M., Gustafsson, G., Garwicz, S., Grander, D., Jonmundsson, G.K., Frost, B.M., Makipernaa, A., Rasool, O., Savolainen, E.R., Schmiegelow, K., Soderhall, S., Vettenranta, K., Wesenberg, F., Einhorn, S. & Heyman, M. (2002) Deletion of the Ink4-locus (the p16ink4a, p14ARF and p15ink4b genes) predicts relapse in children with ALL treated according to the Nordic protocols NOPHO-86 and NOPHO-92. *Leukemia*, **16,** 2037–2045.

Carter, T.L., Watt, P.M., Kumar, R., Burton, P.R., Reaman, G.H., Sather, H.N., Baker, D.L. & Kees, U.R. (2001) Hemizygous p16(INK4A) deletion in pediatric acute lymphoblastic leukemia predicts independent risk of relapse. *Blood*, **97,** 572–574.

Chim, C.S. & Kwong, Y.L. (2006) Adverse prognostic impact of CDKN2B hyper-methylation in acute promyelocytic leukemia. *Leukaemia & Lymphoma*, **47,** 815–825.

Chiosea, S., Krasinskas, A., Cagle, P.T., Mitchell, K.A., Zander, D.S. & Dacic, S. (2008) Diagnostic importance of 9p21 homozygous deletion in malignant mesotheliomas. *Modern Pathology*, **21,** 742–747.

Diccianni, M.B., Batova, A., Yu, J., Vu, T., Pullen, J., Amylon, M., Pollock, B.H. & Yu, A.L. (1997) Shortened survival after relapse in T-cell acute lymphoblastic leukemia patients with p16/p15 deletions. *Leukemia Research*, **21,** 549–558.

Faderl, S., Kantarjian, H.M., Manshouri, T., Chan, C.Y., Pierce, S., Hays, K.J., Cortes, J., Thomas, D., Estrov, Z. & Albitar, M. (1999) The prognostic significance of p16INK4a/p14ARF and p15INK4b deletions in adult acute lymphoblastic leukemia. *Clinical Cancer Research*, **5,** 1855–1861.

Florl, A.R. & Schulz, W.A. (2003) Peculiar structure and location of 9p21 homozygous deletion breakpoints in human cancer cells. *Genes Chromosomes Cancer*, **37,** 141–148.

Forestier, E., Gauffin, F., Andersen, M.K., Autio, K., Borgstrom, G., Golovleva, I., Gustafsson, B., Heim, S., Heinonen, K., Heyman, M., Hovland, R., Johannsson, J.H., Kerndrup, G., Rosenquist, R., Schoumans, J., Swolin, B., Johansson, B. & Nordgren, A. (2008) Clinical and cytogenetic features of pediatric dic(9;20) (p13.2;q11.2)-positive B-cell precursor acute lymphoblastic leukemias: a Nordic series of 24 cases and review of the literature. *Genes Chromosomes Cancer*, **47,** 149–158.

Graf Einsiedel, H., Taube, T., Hartmann, R., Eckert, C., Seifert, G., Wellmann, S., Henze, G. & Seeger, K. (2001) Prognostic value of p16(INK4a) gene deletions in pediatric acute lymphoblastic leukemia. *Blood*, **97,** 4002–4004.

Haller, F., Lobke, C., Ruschhaupt, M., Cameron, S., Schulten, H.J., Schwager, S., von Heydebreck, A., Gunawan, B., Langer, C., Ramadori, G., Sultmann, H., Poustka, A., Korf, U. & Fuzesi, L. (2008) Loss of 9p leads to p16INK4A down-regulation and enables RB/E2F1-dependent cell cycle promotion in gastrointestinal stromal tumours (GISTs). *The Journal of Pathology*, **215,** 253–262.

Hallor, K.H., Staaf, J., Jonsson, G., Heidenblad, M., Vult von Steyern, F., Bauer, H.C., Ijszenga, M., Hogendoorn, P.C., Mandahl, N., Szuhai, K. & Mertens, F. (2008) Frequent deletion of the CDKN2A locus in chordoma: analysis of chromosomal imbalances using array comparative genomic hybridisation. *British Journal of Cancer*, **98,** 434–442.

Heyman, M. & Einhorn, S. (1996) Inactivation of the p15INK4B and p16INK4 genes in hematologic malignancies. *Leukaemia & Lymphoma*, **23,** 235–245.

Kawamata, N., Ogawa, S., Zimmermann, M., Kato, M., Sanada, M., Hemminki, K., Yamatomo, G.,

Nannya, Y., Koehler, R., Flohr, T., Miller, C.W., Harbott, J., Ludwig, W.D., Stanulla, M., Schrappe, M., Bartram, C.R. & Koeffler, H.P. (2008) Molecular allelokaryotyping of pediatric acute lymphoblastic leukemias by high-resolution single nucleotide polymorphism oligonucleotide genomic microarray. *Blood*, **111,** 776–784.

Kees, U.R., Burton, P.R., Lu, C. & Baker, D.L. (1997) Homozygous deletion of the p16/MTS1 gene in pediatric acute lymphoblastic leukemia is associated with unfavorable clinical outcome. *Blood*, **89,** 4161–4166.

Kustanovich, A.M., Savitskaja, T.V., Bydanov, O.I., Belevtsev, M.V. & Potapnev, M.P. (2005) Aberrant expression of tumor suppressor genes and their association with chimeric oncogenes in pediatric acute lymphoblastic leukemia. *Leukemia Research*, **11,** 1271–1265.

Maloney, K.W., McGavran, L., Odom, L.F. & Hunger, S.P. (1998) Different patterns of homozygous p16INK4A and p15INK4B deletions in childhood acute lymphoblastic leukemias containing distinct E2A translocations. *Leukemia*, **12,** 1417–1421.

Mirebeau, D., Acquaviva, C., Suciu, S., Bertin, R., Dastugue, N., Robert, A., Boutard, P., Mechinaud, F., Plouvier, E., Otten, J., Vilmer, E. & Cave, H. (2006) The prognostic significance of CDKN2A, CDKN2B and MTAP inactivation in B-lineage acute lymphoblastic leukemia of childhood. Results of the EORTC studies 58881 and 58951. *Haematologica*, **91,** 881–885.

Moorman, A.V., Ensor, H.M., Richards, S.M., Chilton, L., Schwab, C., Kinsey, S.E., Vora, A., Mitchell, C.D. & Harrison, C.J. (2010) Prognostic effect of chromosomal abnormalities in childhood B-cell precursor acute lymphoblastic leukaemia: results from the UK Medical Research Council ALL97/99 randomised trial. *The Lancet Oncology*, **11,** 429–438.

Mullighan, C.G., Goorha, S., Radtke, I., Miller, C.B., Coustan-Smith, E., Dalton, J.D., Girtman, K., Mathew, S., Ma, J., Pounds, S.B., Su, X., Pui, C.H., Relling, M.V., Evans, W.E., Shurtleff, S.A. & Downing, J.R. (2007) Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature*, **446,** 758–764.

Mullighan, C.G., Heatley, S.L., Danner, S., Dean, M.M., Doherty, K., Hahn, U., Bradstock, K.F., Minchinton, R., Schwarer, A.P., Szer, J. & Bardy, P.G. (2008) Mannose-binding lectin status is associated with risk of major infection following myeloablative sibling allogeneic hematopoietic stem cell transplantation. *Blood*, **112,** 2120–2128.

Ogawa, S., Hangaishi, A., Miyawaki, S., Hirosawa, S., Miura, Y., Takeyama, K., Kamada, N., Ohtake, S., Uike, N., Shimazaki, C., Toyama, K., Hirano, M., Mizoguchi, H., Kobyashi, Y., Furusawa, S., Saito, M., Emi, N., Yazaki, Y., Ueda, R. & Hirai, H. (1995) Loss of the cyclin-dependent kinase 4-inhibitor (p16; MTS1) gene is frequent in and highly specific to lymphoid tumors in primary human hematopoietic malignancies. *Blood*, **86,** 1548–1556.

Perez-Vera, P., Salas, C., Montero-Ruiz, O., Frias, S., Dehesa, G., Jarquin, B. & Rivera-Luna, R. (2008) Analysis of gene rearrangements using a fluorescence in situ hybridization method in Mexican patients with acute lymphoblastic leukemia: experience at a single institution. *Cancer Genetics and Cytogenetics*, **184,** 94–98.

Ramakers-van Woerden, N.L., Pieters, R., Slater, R.M., Loonen, A.H., Beverloo, H.B., van Drunen, E., Heyman, M., Moreno, T.C., Rots, M.G., van Wering, E.R., Kamps, W.A., Janka-Schaub, G.E. & Veerman, A.J. (2001) In vitro drug resistance and prognostic impact of p16INK4A/P15INK4B deletions in childhood T-cell acute lymphoblastic leukaemia. *British Journal of Haematology*, **112,** 680–690.

Savola, S., Nardi, F., Scotlandi, K., Picci, P. & Knuutila, S. (2007) Microdeletions in 9p21.3 induce false negative results in CDKN2A FISH analysis of Ewing sarcoma. *Cytogenetic and Genome Research*, **119,** 21–26.

Schmiegelow, K., Forestier, E., Hellebostad, M., Heyman, M., Kristinsson, J., Soderhall, S. & Taskinen, M. (2010) Long-term results of NOPHO ALL-92 and ALL-2000 studies of childhood acute lymphoblastic leukemia. *Leukemia*, **24,** 345–354.

Sulong, S., Moorman, A.V., Irving, J.A., Strefford, J.C., Konn, Z.J., Case, M.C., Minto, L., Barber, K.E., Parker, H., Wright, S.L., Stewart, A.R., Bailey, S., Bown, N.P., Hall, A.G. & Harrison, C.J. (2008) A comprehensive analysis of the CDKN2A gene in childhood acute lymphoblastic leukaemia reveals genomic deletion, copy number neutral loss of heterozygosity and association with specific cytogenetic subgroups. *Blood*, **113,** 100–107.

Takeuchi, S., Koike, M., Seriu, T., Bartram, C.R., Slater, J., Park, S., Miyoshi, I. & Koeffler, H.P. (1997) Homozygous deletions at 9p21 in childhood acute lymphoblastic leukemia detected by microsatellite analysis. *Leukemia*, **11,** 1636–1640.

Tsuzuki, S., Karnan, S., Horibe, K., Matsumoto, K., Kato, K., Inukai, T., Goi, K., Sugita, K., Nakazawa, S., Kasugai, Y., Ueda, R. & Seto, M. (2007) Genetic abnormalities involved in t(12;21) TEL-AML1 acute lymphoblastic leukemia: analysis by means of array-based comparative genomic hybridization. *Cancer Science*, **98,** 698–706.

Walker, G.J., Flores, J.F., Glendening, J.M., Lin, A.H., Markl, I.D. & Fountain, J.W. (1998) Virtually 100% of melanoma cell lines harbor alterations at the DNA level within CDKN2A, CDKN2B, or one of their downstream targets. *Genes Chromosomes Cancer*, **22,** 157–163.

Woo, H.Y., Kim, D.W., Park, H., Seong, K.W., Koo, H.H. & Kim, S.H. (2005) Molecular cytogenetic analysis of gene rearrangements in childhood acute lymphoblastic leukemia. *Journal of Korean Medical Science*, **20,** 36–41.

Yamada, Y., Hatta, Y., Murata, K., Sugawara, K., Ikeda, S., Mine, M., Maeda, T., Hirakata, Y., Kamihira, S., Tsukasaki, K., Ogawa, S., Hirai, H., Koeffler, H.P. & Tomonaga, M. (1997) Deletions of p15 and/or p16 genes as a poor-prognosis factor in adult T-cell leukemia. *Journal of Clinical Oncology*, **15,** 1778–1785.

van Zutven, L.J., van Drunen, E., de Bont, J.M., Wattel, M.M., Den Boer, M.L., Pieters, R., Hagemeijer, A., Slater, R.M. & Beverloo, H.B. (2005) CDKN2 deletions have no prognostic value in childhood precursor-B acute lymphoblastic leukaemia. *Leukemia*, **19,** 1281–1284.