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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

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Inactivation of the Ink4 gene locus locus on 9p comprising the tumour suppressor gene *p16ink4a* and its neighbours *p14ARF* and *p15ink4b* is common in childhood acute lymphoblastic leukaemia (ALL), but the prognostic significance is controversial. DNA from 230 patients was retrospectively analysed by Southern blotting, single strand conformation polymorphism (SSCP) and sequencing techniques. The results were correlated with clinical characteristics and outcome. One hundred and ninety-four fully analysed patients, similarly treated using the Nordic NOPHO-86 or the current NOPHO-92 protocols, were included in the outcome analysis. Deletions approached a minimally deleted region between the *p16ink4a* and *p15ink4b* genes, making the *p14ARF* gene the most commonly deleted coding sequence. Bi-allelic deletion was associated with high white blood cell count (WBC) ($P < 0.001$), T cell phenotype ($P < 0.001$) and mediastinal mass ($P < 0.001$). Patients with Ink4 locus bi-allelic deletions had an inferior pEFS ($P < 0.01$) and multivariate analysis indicated that bi-allelic deletion of the *p16ink4a* and the *p14ARF* genes was an independent prognostic risk factor ($P < 0.05$). Sub-group analysis revealed a pronounced impact of deletion status for high-risk patients, ie with high WBC. Deletion-status and clinical risk criteria (WBC) could thus be combined to further differentiate risk within the high-risk group. The analysis of the Ink4 locus adds independent prognostic information in childhood ALL treated by Nordic protocols and may help in selection of patients for alternative treatment. *Leukemia* (2002) **16,** 2037–2045. doi:10.1038/sj.leu.2402697
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Introduction

The remarkable improvement in the outcome of childhood acute lymphoblastic leukaemia over the last 20 years has shifted the focus of the treatment from simply curing children with leukaemia to the more complex issue of curing the patient at minimum risk. Since less intensive treatment protocols cured a significant fraction of patients already in the 1970s, the intensification of the therapy has led to the awareness that most protocols are over-treating a large proportion of patients. This is true despite the fact that about 20% of the patients die, most commonly because of relapse of the disease. Although the treatment is highly diversified with regard to dose intensity and accumulated dose with the help of clinical and biochemical risk criteria, there is a need for more powerful tools to identify patients with lower and higher risk at diagnosis. These tools should preferably pick out, both the cases that will experience a relapse of the disease despite favourable prognostic signs at diagnosis, and the high-risk patients that because of a very high risk of relapse even on extremely dose-intensive protocols should be treated by alternative methods, ie allogeneic stem cell transplantation in first complete remission (CR1).

Analyses of genetic alterations in the malignant cells has been included in the stratification of childhood ALL for many years, of which the adverse prognostic impact of the t(9;22) (Ph+) was one of the first connections between mutations and prognosis to be shown.[1] Several other mutations with impact on outcome have since been described: t(4;11), t(1;19), hyperdiploid or hypodiploid karyotype, t(12;21) etc.[2,3] However, most of these fail to have an impact on the treatment of a large proportion of children with ALL for one of two reasons: either the mutation itself is uncommon as with t(9;22) and t(4;11), or it favourably affects the outcome of the children with an already low risk of relapse, such as the t(12;21).[4] The low number of adverse events in this group of patients makes prognostically favourable factors difficult to evaluate.

Deletions on 9p in ALL have been detected by cytogenetic or molecular techniques since 1983.[5–7] The prognostic impact of such deletions has, however, been controversial.[8–11] The mapping of the cell-cycle regulating genes *p16ink4a* and *p15ink4b*, coding for the tumour suppressor protein P16INK4A and its close relative P15INK4B to the minimally deleted region on 9p has for many investigators concluded the search for the tumour suppressor gene in this region.[12,13] The function of these proteins to inhibit the cyclin-dependent kinases 4 and 6 which normally phosphorylate and thereby inactivate critical targets such as the retinoblastoma protein during the G1-phase of the cell-cycle, is easy to reconcile with a role as tumour suppressor protein.[14,15] Later studies have pointed to the probable role of these proteins as mediators of senescence, which is also compatible with a tumour suppressor function.[16,17]

The third coding sequence of the locus, *p14ARF*, was firstly discovered as an alternative transcript of the *p16ink4a* gene, but was later found to code for an unrelated protein through its unique property of differential use of an alternative first exon spliced on to the second exon in a different reading-frame.[18,19] The function of the P14ARF protein may be equally important as a tumour suppressor activity in that it interferes with p53 turnover by binding to and inactivating the MDM-2 protein, which in turn targets p53 for degradation. Furthermore, ARF knockout mice develop much the same types of tumours as the *p16ink4a* exon 2 knockouts, a mutation that also disrupts *p14ARF* exon 2.[20] Indeed, recent studies with transgenic mice carrying inactivating mutations of the *p16ink4a* gene only, show a less pronounced but still signifi-

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cant phenotype predisposing these animals for tumour formation.[21]

The only Ink4 locus gene that has actually been shown to be selectively targeted by somatic mutations (loss of one allele and inactivating point mutation of the other) in ALL is the *p16ink4a* gene,[22] but the *p15ink4b* gene has been shown to be inactivated by methylation[23] and some intragenic mutations in exon 2 of *p16ink4a/p14ARF* are likely to impair both coding sequences.[15,24,25] Furthermore, most inactivating mutations are homozygous deletions that in an absolute majority of the cases include the *p14ARF* sequence as well as one of the neighbouring genes[26] (and the present investigation).

The prognostic impact of Ink4-locus (*p16ink4a, p15ink4b* and *p14ARF*) inactivation in childhood ALL has been studied by several groups with conflicting results: The BFM group and studies from St Jude have not shown any correlation between Ink4 locus inactivation and outcome,[27,28] while our group, as well as studies from southern United States and from Australia have shown an adverse prognostic impact.[25,29–31] Some of the drawbacks of the earlier studies have either been their size or lack of homogenous treatment.

The present study was undertaken to confirm or refute the significance of Ink4 locus inactivation as a prognostic factor in childhood ALL in patients treated according to Nordic ALL protocols (NOPHO-86 and the currently used NOPHO-92), with the secondary aim to evaluate whether analysis of this locus could contribute valuable information in the risk stratification of childhood ALL.

Materials and methods

Patients

Diagnostic bone marrow or peripheral blood samples were obtained from paediatric oncology units in the Nordic countries that had collected and stored them for future analyses. Sufficient material was available for analysis from 230 patients diagnosed between 1987 to 1997. Results concerning the status of the *p16ink4a* and p*15ink4b* genes in 79 patients have been previously published.[25] The NOPHO study group has designed protocols that are uniformly used throughout the Nordic countries for patients with ALL, 1 year to $<$15 years of age at diagnosis and a non-mature B cell immunophenotype. Infants $<$1 year (6 patients) and mature B-cell ALL (7 patients) have been treated according to various other protocols. These patients, as well as patients $\geqslant$15 years at diagnosis (9 patients), were excluded from the outcome analysis as was a patient with t(9;22) who received a bone marrow transplantation in CR1. Of the remaining 207 patients, 194 could be fully analysed for all three coding sequences. Fifty-four were diagnosed from 1987 to 1991 and were treated according to the protocol NOPHO-86, 140 were diagnosed from 1992 to 1997 and the subsequent NOPHO-92 protocol was used to treat these patients.[32] A total of 735 patients between 1 and 15 years of age were diagnosed with non-B cell ALL in the catchment regions of the Nordic centres contributing material to the study from September 1987 to October 1997 (months of the first and last included patient). This cohort constitutes the whole population of children with ALL in the Nordic countries from which the sample was taken.

The clinical characteristics of the 194 (as well as of the non-analysed) patients are shown in Table 1. The median follow-up for NOPHO-86 patients was 119.5 months (range 96–147) and for NOPHO-92 patients 62.5 months (range 26–95).

Treatment

The different treatment protocols (NOPHO-86 and NOPHO-92) are described in detail in Ref. 32. Both protocols consist of a backbone of a remission induction based on prednisolone, doxorubicin, vincristine and L-asparaginase followed by consolidation with high-dose methotrexate infusions and maintenance with oral methotrexate and 5-mercaptopurine. For patients in higher risk groups, early and late intensification elements are added. The early intensification consists of cyclophosphamide and blocks of low-dose cytarabinoside and the late intensification element is similar to the induction phase. The dose intensity of the consolidation, the addition of a late intensification phase as well as the duration of the maintenance therapy depends on the risk group. Approximately 25% of the patients received cranial irradiation in NOPHO-86 and approximately 7% in NOPHO-92. In the latter protocol, high-dose (5 or 8 g/m^2 body surface) methotrexate infusions and, for high-risk patients, additional courses of high-dose cytarabinoside (2 g/m^2) has replaced cranial irradiation as CNS prophylaxis for many patients. In NOPHO-92, no patients $<$5 years old receive cranial irradiation. For patients with poor prognostic features at diagnosis, eg t(9;22), there is an option in both protocols of bone marrow transplantation in CR1.

Preparation of cells and extraction of DNA

Mononuclear cells were separated from leukaemic peripheral blood or bone marrow by separation on Lymphoprep (Nycomed, Oslo, Norway) and frozen in liquid nitrogen until the diagnosis and the purity of the cell population was known. Cells were then thawed and, in cases in which flow cytometry analysis showed significant contamination with normal cells, malignant cells were purified by positive or negative panning.[33] All preparations used for DNA extraction were more than 85% pure. In some cases with very pure leukaemic cell populations, DNA extracts were prepared directly, without separation. DNA extraction, digestion, transfer and hybridisation have been previously described.[7] The signal intensity was assessed by visual inspection and measured by scanning densitometry using an Ultroscan XL (Pharmacia LKB, Bromma, Sweden). In cases of decreased, but not absent hybridisation signal, ratios between the signal for the Ink4 locus gene in question and a control probe was calculated and compared with normal cell DNA blotted on to the same filter. The ratio for the normal control was set to 1.0. A patient was considered to have a bi-allelic deletion if the standardised ratio was $<$0.30 (the absolute majority of these patients had ratios less than 0.15), retention of both alleles if the ratio was $>$0.85 and mono-allelic deletion if the ratio was in between these values. Most latter cases had ratios clustered between 0.40 and 0.65. Furthermore, for a number of the cases assessed as mono-allelic deletion, LOH data supporting this finding was available from previous studies.[10,22,25]

Cytogenetic analysis

Results from the reviewed diagnostic cytogenetic analysis were obtained from the cytogenetic working group of NOPHO, reported to the Nordic childhood ALL registry.

Table 1 Clinical characteristics of the fully analysed patients compared with all other ALL patients diagnosed in the participating Nordic centres over the same time period. Percentage of patients shown

Parameter	*0 allele*[a] *n = 55*	*1 allele n = 30*	*2 alleles n = 109*	*P value*[b]	*Analysed patients n = 194*	*Not analysed patients n = 735*	*P-value*[c]
Age				0.129			0.004
1–<5	38	40	55		48	60	
5–<10	31	33	28		30	26	
⩾10	31	27	17		22	14	
Sex				0.586			0.068
Male	62	63	56		59	51	
Female	38	37	44		41	49	
CNS leukemia				0.571			0.484
Yes	4	3	2		3	2	
No	96	97	98		97	97	
Missing					0	1	
Immuno-phenotype[d]				<0.001			0.020
B-precursor	67	90	93		86	90	
T cell	33	7	7		14	9	
Incomplete analysis		3			0	1	
Lymphomatous ALL[e]				0.007			0.244
Yes	24	14	5		11	8	
No	76	86	95		89	92	
Mediastinal mass				<0.001			0.001
Yes	29	7	8		14	6	
No	71	93	92		86	93	
Missing					0	1	
WBC[c]				<0.001			0.001
<10	24	26	48		36	50	
10–<50	25	37	37		35	32	
⩾50	51	37	15		29	18	
Hb				0.014			0.013
<100	60	60	85		73	82	
⩾100	35	40	13		25	17	
Missing information	5	0	2		2	1	
Blasts day 29[e]				0.744			0.538
0–<5	81	86	89		86	84	
5–<25	3	5	1		3	4	
>25	0	0	0		0	1	
Missing	16	9	10		11	11	
Risk-group				<0.001			0.009
SR	13	13	35		25	34	
IR	20	44	44		37	36	
HR (1+2)[f]	45	30	13		25	24	
VHR[g]	22	13	8		13	6	
Hyperdiploid karyotype[h]				0.001			<0.001
Yes	0	19	17		14	27	
No	100	81	83		86	73	

[a]Refers to patients with bi-allelic deletions according to variable A.
[b]The *P* value refers to the comparison of patients carrying bi-allelic deletions *vs* patients without deletions or with mono-allelic deletions (patients with mono-allelic deletions and normal gene dose are grouped together).
[c]Comparison of the sampled and analysed patients *vs* the non-analysed population of children with ALL from the participating centres.
[d]'Lymphomatous feature ALL' and blast cells on day 29 after diagnosis were only recorded for NOPHO-92 patients.
[e]The WBC stratification is identical to that of the treatment protocols (patients change from 'standard-risk' to 'intermediate risk' if WBC is ⩾10 and <50 and from 'intermediate risk' to 'high risk' if WBC is ⩾50.
[f]High-risk groups 1 and 2 (for definition of these groups, see Ref. 32).
[g]Very high-risk group (for definition of this group, see Ref. 32).
[h]>51chromosomes, based on the number of patients with successful cytogenetic analysis.

Probes for Southern hybridisation

The filters were sequentially hybridised with the following probes: Three different probes were used for detection of the Ink4-locus: (1) *p15ink4b* exons 1 and 2; (2) *p16ink4a* exons 1 and 2; (3) the E1β exon from *p14ARF* gene. The *p16ink4a* hybridisations were performed with a full-length cDNA probe, kindly provided by Dr D Beach (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA). The *p15ink4b* probe was a cDNA that comprised the two exons of the gene and additional flanking sequences, also provided by Dr D Beach. The hybridisation patterns of these probes have been described previously.[22] The Southern probe used for the E1β exon (736 bp) was obtained from PCR reactions using the following primers: 5′-TCCCAGTCTGCAGTTAAGG-3′ and 5′-GTCTAAGTCGTTGTAACCCG-3′.[19] Several probes were used as controls: (1) a 1 kb cDNA fragment corresponding to the γ-*IFN* gene on chromosome 12;[34] (2) a 1.6 kb fragment including the 5′ region of the *Bcl-2* oncogene on chromosome 18;[35] (3) The *Bcl-1* probe located on chromosome 11 band q13;[36] and (4) a 3 kb *EcoRI* fragment from chromosome 5 corresponding to D5S78.[37] Several filters were hybridised with

more than two probes, but the *Bcl-1* and D5S78 probes were hybridised to all filters.

Single-strand conformation polymorphism analysis (SSCP)

Malignant and, when available, normal cell DNA was amplified with regard to exons 1 and 2 of the *p15ink4b* and *p16ink4a* genes and the E1β exon of the *p14ARF* gene. PCR conditions and a description of the SSCP method have been previously published for the *p15ink4b* and *p16ink4a* genes.[25] The E1β exon of the *p14ARF* gene was amplified by PCR using the primers and conditions previously described.[38] Before loading on to SSCP gels the PCR products were digested with *Bgl*I. Four different conditions were used: room temperature ±50% glycerol and +4°C ±50% glycerol. SSCP samples from the other exons were analysed in glycerol-containing gels and run at room temperature with an external cooling fan placed in front of the plates. After electrophoresis, gels were transferred to Whatman 3MM paper (Merck Eurolab, Stockholm, Sweden), dried and autoradiographed on X-ray film.

Nucleotide sequencing

Nucleotide sequencing of exons 1 and 2 from both the *p15ink4b* and *p16ink4a* genes was performed as previously described.[22]

Statistical analysis

Statistical analyses were performed using the SPSS statistical software (SPSS: Base 10.0 and Advanced Statistics 10.0 for Windows; Chicago, IL, USA). The chi-square test according to the likelihood ratio method was applied to test for significance between distributions of different subgroups. Cox multivariate proportional hazards regression analyses were performed to evaluate prognostic factors. The life-table method was used to generate graphs and the Kaplan–Meier method was used to estimate probability of event-free survival (p-EFS). The log-rank test was used to compare prognoses between different subgroups. Odds ratios were calculated using a World Wide Web utility.[39] The patients were followed from diagnosis until the time of the first adverse event or the end of the follow-up period, which was defined as 31 December 1999. Events were defined as induction death, death in complete remission, relapse and second malignant neoplasm. Induction death was considered an event at the time point zero. A significance level of 0.05 was used in all statistical analyses and all tests used were two-tailed.

Results

Study population in relation to the Nordic population of childhood ALL

The Nordic Society for Pediatric Hematology and Oncology (NOPHO) manages the Scandinavian ALL registry, which is population-based and regularly checked against national cancer registries. Given these pre-conditions any parameter measured in the leukaemic population could be extrapolated to prevalence/incidence rather than sample frequency, once the characteristics of the sample are known. We therefore wanted to find out how representative the analysed material was for the ALL population. The clinical characteristics of the analysed patients and the background population of children with ALL from the participating centres are listed in Table 1. When comparing the non-analysed patients with the study patients there was no significant difference regarding sex, CNS involvement, the prognostically important translocations t(9;22), t(4;11) (not shown) and lymphomatous features of the disease (recorded only for the NOPHO-92 patients) and percentage of blasts in the bone marrow at day 29 after the start of treatment. The study population, however, was significantly older $P < 0.01$), had higher WBC ($P < 0.01$) and higher Hb values ($P < 0.05$), more often presented with T cell phenotype ($P < 0.05$) and a mediastinal mass ($P < 0.01$) and were less likely to have a hyperdiploid karyotype ($P < 0.001$) than the background population. Consequently, the study population was also more likely to be classified into higher risk groups ($P < 0.01$).

At the time of follow-up, 145 out of 194 patients were in CR1 (35 patients from NOPHO-86 and 110 patients from NOPHO-92); 45 patients had relapsed (19 NOPHO-86 patients and 26 NOPHO-92 patients); two patients had died during induction (both NOPHO-92 patients) and two patients had died in continuous complete remission (CCR) (both NOPHO-92 patients). A total of 162 patients were alive at follow-up (41 NOPHO-86 patients and 121 NOPHO-92 patients).

Southern hybridisation and the frequency and prevalence of deletions

Leukemia cell DNA from 230 patients was extracted and analysed by Southern hybridisation. Thirty-six patients were excluded from the outcome analysis for the reasons given above (six infants, seven B cell ALL, nine patients who were $\geqslant$15 years of age at diagnosis, one t(9;22) primarily treated by bone marrow transplantation and 13 not fully analysed). None of the six infants (0%), 3/8 (38%) of the B cell ALL, 2/9 (22%) of the $\geqslant$15-year-olds and the patient with t(9;22) carried bi-allelic deletions of the Ink4 locus. It is noteworthy that bi-allelic deletions occur in all sub-groups of ALL with the exception of infants.

The gene dose of the *p16ink4a* gene was determined in 207 patients, 206 patients were analysed for *p15ink4b* and the E1β exon, coding for the first exon of the *p14ARF* gene could be analysed in 194 cases. Bi-allelic deletion or rearrangement of at least one exon of the *p16ink4a* gene was detected in 50 patients (24%) and the corresponding figure for the *p15ink4b* gene was 37 (18%). Out of the 194 cases that could be fully analysed with regard to the *p14ARF* gene, 53 patients (27%) had bi-allelic deletions. Mono-allelic deletions of at least one of the coding exons were detected in 31 (16%) of the fully analysed patients. In the 79 previously published patients, mono-allelic deletion status was also confirmed with LOH studies using RFLP or microsatellite analysis.[10,22,25] Thus, deletions approached a minimally deleted region between the *p16ink4a* and *p15ink4b* genes both from the telomeric and the centromeric sides, making the *p14ARF* gene the most commonly deleted coding sequence (Figure 1b).

Correcting for sample bias towards more highly cellular ALL cases (high WBC values) and T cell phenotype yielded an estimation of deletion prevalence in an unselected ALL population of 25% for a bi-allelic deletion in any of the coding sequences included in the Ink4 locus.

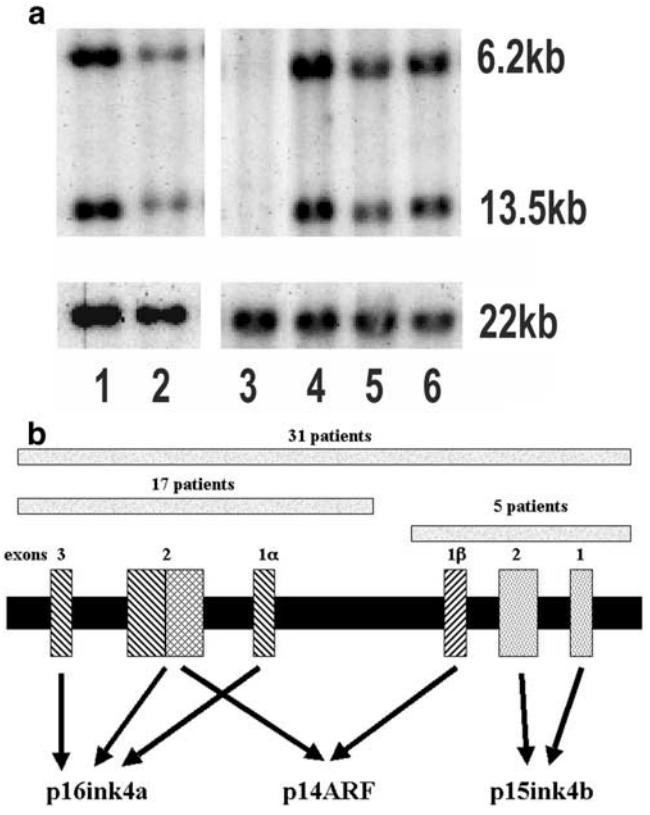

Figure 1 (a) Southern hybridisation of leukaemic cell DNA from ALL patients digested with *Hin*dIII and hybridised with probes for *p16ink4a* exons 1 (6.2 kb) and 2 (13.5 kb). D5S78 (22 kb) was used as a control probe. DNA from the patients represented in lanes 1 and 6 and normal DNA from a healthy control in lane 6 are not deleted for any of the probes; patient 3 carries bi-allelic deletions of all Ink4 locus genes, including *p16ink4a*. DNA from patients 2 and 5 show mono-allelic deletion of *p16ink4a* exons 1 and 2. (b) Schematic presentation of the Ink4 locus with the three coding sequences: *p16ink4a*, *p14ARF* and *p15ink4b* and the different bi-allelic deletion patterns represented in the study. Leukaemic cell DNA from 31 patients was bi-allelically deleted for the entire locus, 17 patients carried deletions inactivating *p16ink4a* and *p14ARF* by the deletion of *p16in4a* exons 1 and 2, whereas five patients had deletions limited to *p15ink4b* and *p14ARF* (exon 1β-sequences). One patient fell outside of this pattern with bi-allelic deletion/rearrangement of both *p16ink4a* and *p15ink4b* with sparing of *p14ARF* sequences (see text).

Essentially three different bi-allelic deletion patterns occurred (Figure 1b): (1) deletion of the entire locus (31 patients); (2) deletion of the *p16ink4a* and *p14ARF* genes with sparing of *p15ink4b* sequences (17 patients); (3) deletion of *p15ink4b* and *p14ARF* with sparing of *p16ink4a* (five patients). A single patient formed an exception. This patient had a complex rearrangement with deletion of one allele of the entire locus and rearrangement of the remaining *p16ink4a* (exon 1) and *p15ink4b* allele, but no visible rearrangement in the E1β exon and thus the *p14ARF* sequences appeared intact.

SSCP analysis and sequencing

Out of the 31 patients with mono-allelic deletion of a part of the Ink4 locus, 21 patients could be successfully analysed by SSCP and/or nucleotide sequencing (data not shown). The remaining 10 patients could not be analysed due to shortage of material or technical problems. Two patients with intragenic mutations were detected: one patient had a nucleotide exchange in *p16ink4a*/*p14ARF* exon 2, resulting in a nonsense mutation with regard to the *p16ink4a* gene and a conservative amino acid substitution with regard to the P14ARF protein. The other patient had a complex substitution of seven nucleotides for six new nucleotides in exon 1 of the *p16ink4a* gene, resulting in a frame-shift and a premature stop within the *p16ink4a* reading frame. Both these intragenic mutations have been previously described[22,25] All other SSCP/sequence analyses were normal.

Mutation status in relation to clinical characteristics

The clinical characteristics of the patients in relation to mutation status are described in Table 1. There was no significant correlation between deletion/mutation status and age, sex, CNS involvement at diagnosis and the level of blasts in the bone marrow at day 29 before start of treatment (defined only for NOPHO-92 patients), whereas bi-allelic deletion of the Ink4 locus was associated with higher WBC ($P < 0.001$), higher Hb ($P < 0.05$), T cell phenotype ($P < 0.001$) as well as the presence of a mediastinal mass at diagnosis ($P < 0.001$) and 'lymphomatous feature ALL' (defined only for NOPHO-92 patients) ($P < 0.01$). Taken together, patients with homozygous deletion within the Ink4 locus were more likely to be assigned to 'high-risk' treatment groups than patients without deletions ($P < 0.001$) although patients with deletions were found in all risk groups (Table 1). There were no patients in the studied population (non-B cell ALL 1–14 years at diagnosis) who had a t(4;11) translocation. Three patients had a t(9;22) translocation (two patients with homozygous deletion of the whole Ink4 locus and one without deletion). With regard to ploidy, there was an inverse correlation between hyperdiploid karyotype and homozygous deletion of the Ink4 locus ($P < 0.01$). This was also true when the B-precursor ALL group was analysed separately.

Analysis of clinical outcome in relation to mutation status

The functional significance of deletion of parts of or the entire Ink4 locus is not fully understood. Since there may be synergistic effects from losing more than one of the Ink4 locus genes (ie affecting both the pRb and the p53 pathways), we investigated the different types of deletions as well as combinations of deletions for prognostic impact by survival analysis. A systematic analysis of all possible bi-allelic deletions/rearrangements in the locus showed that essentially all deletion patterns were strongly linked to a decrease in p-EFS with one exception: out of the five patients with a combined deletion of the *p15ink4b* and *p14ARF* genes without apparent interruption of *p16ink4a* sequences, four were in continuous complete remission at the time of follow-up.

In order to obtain valuable and possibly cost-effective prognostic markers we decided to use two groupings for the subsequent survival analyses: Variable A divided the patients into bi-allelic deletion of any of the three genes *vs* mono-allelic deletion and normal gene dose taken together. This variable was chosen since the relative importance of the Ink4 locus genes is still unclear. Variable B uses the bi-allelic deletion of *p16ink4a* and *p14ARF*-coding sequences *vs* patients with retention of at least one allele of one of these sequences. This deletion pattern leads to the inactivation of both the Rb and

p53 pathways, which in experimental systems have shown synergistic effects supporting the rationale for this grouping.[21] The strongest impact was recorded for variable B: (pEFS for the group with deletion 0.58, s.e. 0.07 and for the group without deletion 0.77, s.e. 0.04; log-rank: $P < 0.001$; odds ratio (OR) 3.31, 95% confidence interval (CI) 1.62–6.76), whereas the impact of variable A was slightly less pronounced: (pEFS for the group with deletion 0.62, s.e. 0.07 and for the group without deletion 0.76, s.e. 0.05; log-rank: $P < 0.01$; OR: 2.55, 95% CI 1.28–5.06) (Figure 2).

When the patients were sub-divided between the two treatment protocols, the outcome was significantly worse for patients with bi-allelic deletion treated with the NOPHO-92 protocol (log-rank: $P = 0.0038$; OR 2.89, 95% CI 1.23–6.81 for variable A, and log rank: $P = 0.0008$; OR 3.80, 95% CI 1.57–9.16 for variable B) (for pEFS values see Figure 3b). The same trend existed for the NOPHO-86 protocol, but did not reach statistical significance (log-rank: $P = 0.23$; OR 1.08, 95% CI 0.56–5.85, and log-rank: $P = 0.17$; OR 2.45, 95% CI 0.71–8.51 for variables A and B, respectively) (for pEFS values see Figure 3a). With the considerable difference in the size of the sample, this discrepancy may be largely due to a difference in statistical power, but it is noteworthy that the prognosis for patients with bi-allelic deletion does not seem to have improved with the introduction of the new protocol. The improvement in treatment outcome between protocols (overall 5-year pEFS increase from 0.60 to 0.78 in the study population) was thus not evenly distributed between patients with and without deletions, but occurred mainly in the group of patients without deletions. However, when patients with and without deletions were grouped according to protocol, the comparison (regarding variable B) between NOPHO-86 and NOPHO-92 patients reached statistical significance neither for the group with deletion (log rank $p = 0.69$) nor for patients without deletions (log rank $P = 0.12$) (not shown).

Addition of the information obtained from the SSCP and sequence analyses to the outcome variable (variable A) did not add any new prognostic information.

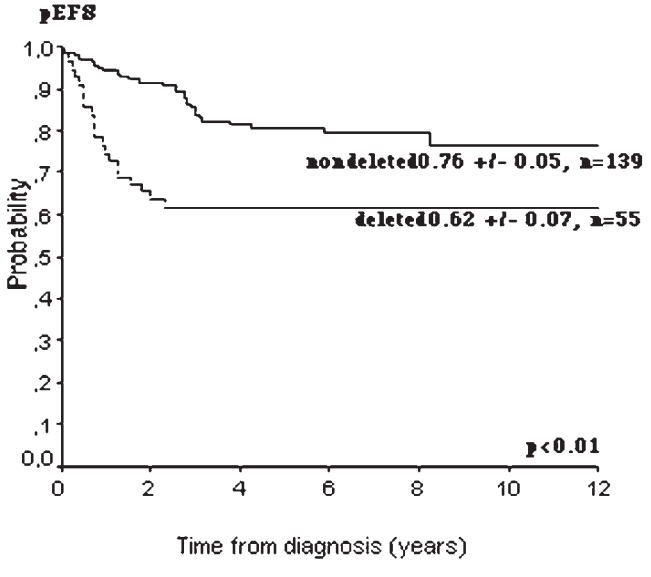

Figure 2 Life-table analysis including all 194 patients (NOPHO-86 and NOPHO-92 patients together). Variable A (any bi-allelic deletion within the Ink4 locus *vs* all others) is used to group the patients.

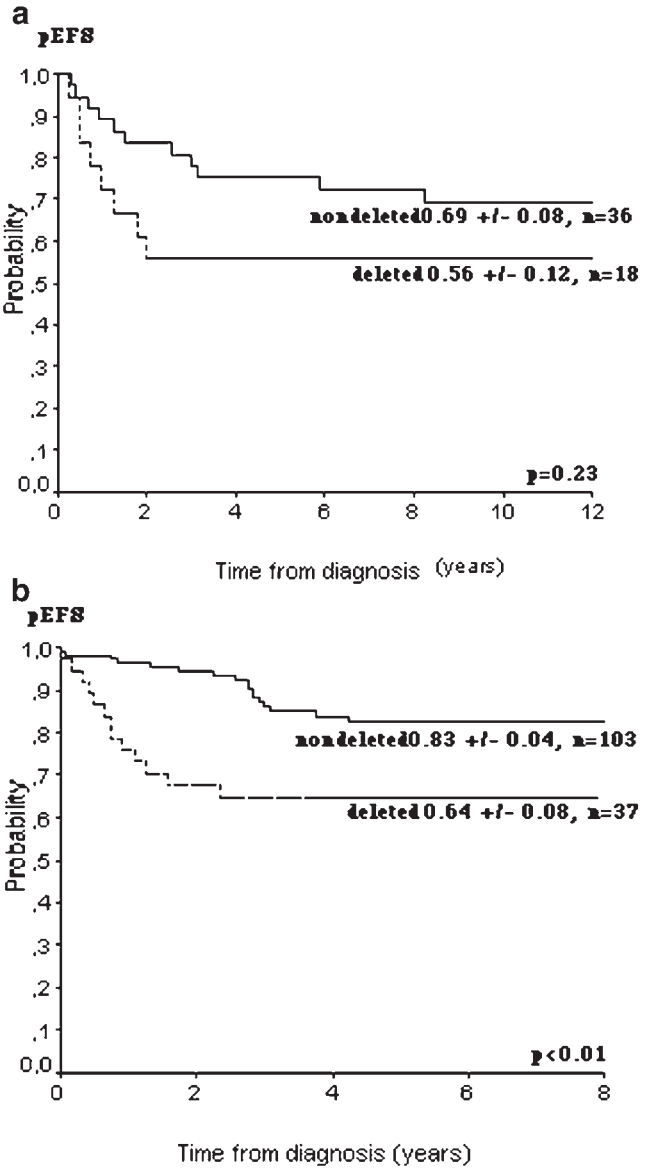

Figure 3 Life-table showing (a) NOPHO-86 and (b) NOPHO-92 protocol patients separately (variable A).

Cox multiple regression (multivariate) analysis

The following clinical parameters were entered into a model using Cox regression analysis according to the Wald backward method: age, sex, involvement of the CNS at diagnosis, the presence of a mediastinal mass at diagnosis, immunophenotype (T cell *vs* B-precursor), lymphomatous ALL, WBC (<10, 10–<50, ⩾50) and treatment protocol (NOPHO-86, NOPHO-92). At first, the deletion variables A and B were tested pairwise against the clinical parameters. Both deletion variables were found to have a stronger impact on outcome than the tested clinical parameters, with the exception of WBC. This was true for both the whole patient material and when only the NOPHO-92 protocol patients were included. Significance remained in the bi-variate comparison with WBC for variable B, but not for variable A for the whole material, as well as when the NOPHO-86 patients were excluded ($P < 0.05$).

Secondly, all parameters were added into a multi-variate

model including all variables listed above. Lymphomatous ALL was removed from the model when the whole patient material was analysed, since this variable was only defined for NOPHO-92 patients. Variable B remained statistically significant ranking after WBC both in the analysis of the whole patient material and when only NOPHO-92 patients were included ($P < 0.01$ and $P < 0.01$, respectively). The same was true for variable A, but at a lower level of significance ($P < 0.05$ for both analyses). Thus, bi-allelic deletion of the Ink4 locus constitutes an independent prognostic factor both in the whole patient material and in the currently used NOPHO-92 protocol.

When patients carrying deletions were analysed separately, only WBC reached statistical significance as a prognostic marker both in the analysis of the whole patient material and when only NOPHO-92 patients were included ($P < 0.05$ and $P < 0.05$). In the analysis of patients without Ink4 locus deletions, WBC and immunophenotype reached statistical significance in both the whole patient material ($P < 0.001$ and $P < 0.01$, respectively) and in the analysis of NOPHO-92 patients ($P < 0.01$ and $P < 0.01$).

We refrained from including cytogenetic parameters in this Cox regression model for two reasons: firstly, the number of patients with failed cytogenetic analysis is fairly high (45/194, 23%); secondly, a clonal abnormality could be detected only in about 55% of the patients.

Sub-groups of patients: clinical outcome with regard to deletion status

The distribution of patients carrying deletions in the leukaemic cells in the material as well as the Cox regression analyses suggests co-variation between deletion status and higher WBCs, which is also linked to the risk stratification in the NOPHO protocols as in all modern treatment regimens. We therefore wanted to explore whether Ink4 locus analyses could help identify sub-groups of patients with worse prognosis in different strata of WBC. In agreement with the overall analysis, there were trends for a worse outcome for patients with deletions in all strata of WBC, but this trend was considerably more pronounced for higher WBC values. When the sub-set of patients with WBC >100 was analysed, patients with deletions fared worse than patients without deletions regarding both variables, but the difference was significant only for variable B ($P < 0.05$ and $P = 0.07$, respectively). The same was true for the corresponding analysis including only NOPHO-92 patients, ($P < 0.05$ and $P = 0.10$, respectively) (Figure 4).

Since deletion status co-varied with immunophenotype and Ink4 locus deletion was associated with T cell phenotype, survival analyses were performed also with regard to T cell and B-precursor cell sub-groups. Deletion status was a significant predictor of relapse for T cell ALL ($P < 0.05$, variable B and variable A). Concerning the B-precursor ALL group the difference only reached statistical significance for variable B ($P < 0.05$ and $P = 0.06$, respectively). When only NOPHO-92 patients were included in the analysis, significance remained for B-precursor cell patients ($P < 0.05$, both variables), but not for T cell patients ($P = 0.28$, both variables). These differences in significance level between the immunophenotype groups are in all likelihood due to differences in statistical power, since the pEFS values were similar for T cell and B-lineage patients in the respective deletion group. Thus, deletion status seems to be an adverse prognostic factor for both T cell and B-lineage ALL.

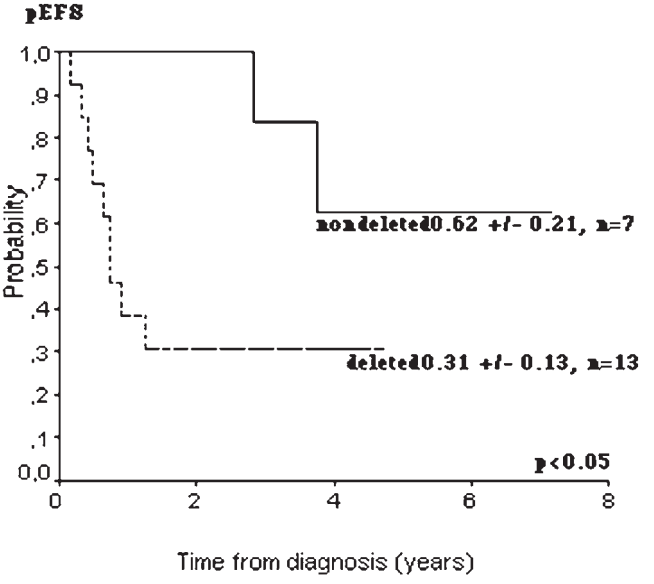

Figure 4 NOPHO-92 patients with WBC values in excess of 100. Life-table analysis comparing patients with bi-allelic deletions according to variable B (*p16ink4a + p14ARF*) *vs* all other patients.

Discussion

The purpose of this multi-centre effort in the Nordic countries was to determine whether Ink 4 locus mutations contribute prognostic information for patients uniformly treated according to Nordic protocols. Although there is some sample bias in the natural selection of patients that yield enough surplus cells to store leukaemic material in a cell bank, the sample size and composition should be sufficient to answer the question. The bias towards highly cellular high-risk cases, which accumulate more events, facilitates the analysis. Complete knowledge of clinical data for the background population of children with ALL makes meaningful comparisons possible despite the sample bias.

The deletion frequency in this material is in agreement with other ALL studies with approximately 30% of the patients carrying bi-allelic deletions and around 10–15% mono-allelic deletion, as is the co-variation between bi-allelic deletion and high-risk features such as high WBC values and T cell phenotype.[28–31] The correction for high WBC and T cell ALL yielded a calculated deletion frequency in unselected ALL cases of about 25%, which should be ideal for a factor that identifies poor outcome in a population, which has about 25% primary adverse events.

The outcome analysis clearly shows the worse prognosis for patients with bi-allelic deletions compared to those with mono-allelic deletions or normal gene dose. This is in agreement with our previous findings and with some other investigators,[29–31] but clearly differs from other studies.[27,28] The reasons for this discrepancy are not obvious. In all studies, patients with Ink4 locus deletions are described as having much the same risk features (ie an over-representation of high WBC, T cell phenotype etc). Discordance in the outcome analysis may be due to differences in the choice of treatment regimen based on variation in the stratification of the patients or possibly differences in treatment outcome in sub-groups of patients.

In judging the value of Ink4 deletions as a prognostic marker, it does not seem cost-effective to include SSCP or

sequencing in the analysis, and assessment of gene dose could probably be limited to the *p16ink4a* gene. Patients with functional inactivation of both *p16ink4a* and *p14ARF* in their leukaemic clone for some reason seem to fare worse, which would indicate that these two coding sequences should be included. However, all *p14ARF* deletions that exclude *p16ink4a* are associated with *p15ink4b* deletions and these patients (although few in number) fare no worse than average. The problem in selecting patients who can be analysed because of the excess material needed, may be addressed in two ways: deletions could be analysed on a single cell level by FISH, but that would possibly reduce the sensitivity of the assay because of the need for larger probes. The other possibility is to use quantitative PCR techniques, of which real-time PCR seems most attractive.

The study may shed some light on the relative importance of the different genes in the Ink4 locus. The deletion data support a role in leukaemogenesis for the *p14ARF* gene in that it maps to the minimally deleted region, but this region is very close to exon 1 of the *p16ink4a* gene. There is no evidence for or against the possibility that this gene is also functionally compromised by the centromeric deletions, which inactivate *p14ARF* and *p15ink4b*. All current data on intragenic mutations support a tumour suppressor function for the *p16ink4a* gene. The fact that deletions including the *p14ARF* sequences with intact *p16ink4* gene sequences are not prognostically deleterious singles out the *p16ink4a* gene as the prognostically important gene. There may, however, be synergistic effects with regard to sensitivity to chemotherapeutic agents in the inactivation of both the pRb and the p53 pathways by way of a combination of deletions of the *p16ink4a* and *p14ARF* genes.[21] Whether *p15ink4b* plays any role at all with regard to prognosis is unclear even if the methylation data suggest a role in leukaemogenesis. If anything, the data from the present investigation speak against it.

It is noteworthy that the group of patients with deletions has almost superimposable survival curves with the NOPHO-86 and the NOPHO-92 protocols. Even if the differences are not statistically significant, it is evident from the shape of the graphs that the course of the disease has altered more for patients without bi-allelic deletions with the advent of the new protocol. This result is perhaps the strongest reason to explore the possibilities of alternative therapy for patients with bi-allelic deletions when new treatment strategies are devised.

It is also important to point out that although deletions are highly prevalent in T cell disease, the prognostic value of the deletion analysis is at least equal in the B-precursor cell group of patients. Indeed, comparisons including only B-precursor cell patients produce lower *P* values, but this is probably due to superior statistical power.

As a prognostic marker, deletion status seems to have a higher impact in higher risk groups, especially for patients with high WBC values. This finding indicates that alteration in stratification or selection of patients for 'up-grading' probably should be limited to these higher risk groups. A further indication supporting this conclusion is the fact that out of the participating centres, the analysed cohort makes up approximately 20% of the total number of patients from lower risk groups and up to half of the patients for the corresponding high-risk groups.

The multivariate analysis confirms that deletion status is an independent prognostic factor, but that it also co-varies with WBC. It was therefore logical to try to identify strata of WBC values in which deletion status contributed additional prognostic information. The striking differences in survival for patients with high WBC values with regard to deletion status could form a basis for selection criteria for alternative treatment (ie allogeneic stem cell transplantation in CR1). Further evaluation of Ink4 locus deletions as a stratification tool in a prospective setting is warranted.

In conclusion, the study of the Ink4 locus in leukaemic cells has added to our knowledge in the field of basic cell-cycle regulation and the pathways regulating it. The present investigation also suggests that this understanding together with carefully collected epidemiological data may translate into a tool capable of helping the clinician in the choice of treatment strategies for the patient.

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