

Expression of *HOX11* in childhood T-lineage acute lymphoblastic leukaemia can occur in the absence of cytogenetic aberration at 10q24: a study from the Children's Cancer Group (CCG)

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Clonal genetic aberrations in tumour cells provide critical information for the development of new diagnostic and therapeutic strategies for patients. In paediatric T-cell acute lymphoblastic leukaemia (T-ALL) chromosomal translocations are present in 30–35% of cases. *HOX11* and the closely related *HOX11L2* genes play a key role in T-ALL. *HOX11* is aberrantly activated by either of the two chromosomal translocations, t(7;10) and t(10;14). In this study, *HOX11* expression levels were measured by real-time quantitative reverse-transcriptase polymerase chain reaction. We show that leukaemic blasts from 15/76 (19.7%) paediatric T-ALL patients expressed the *HOX11* gene at high level and 22/76 (28.9%) at low level, yet the reported frequency for chromosomal rearrangement of 10q24 is 4–7%. Direct cytogenetic analysis revealed that only 2/16 specimens that showed *HOX11* expression exhibited abnormalities at 10q24. These results confirm and extend our previously published findings, and implicate mechanisms other than gross chromosomal translocations for the deregulation of *HOX11*. Analysis of clinical outcome for the whole study group showed a trend for better outcome for patients with leukaemic blasts expressing *HOX11* at high level. A statistically significant difference in clinical outcome was found in a subgroup of 20 patients treated for high-risk disease on CCG-1901 from the Children's Cancer Group, where *HOX11* expression in leukaemic blasts conferred a prognostic advantage ($P=0.01$).

Leukemia (2003) 17, 887–893. doi:10.1038/sj.leu.2402892

Keywords: childhood T-ALL; *HOX11*; quantitative RT-PCR; cytogenetics

Introduction

As a group, the acute leukaemias are the most common form of cancer in children, accounting for approximately one-third of all juvenile neoplasms (age < 16 years) in developed countries. The majority of these cases are classified as acute lymphoblastic leukaemia (ALL) with about 15% of these being of T-cell phenotype (T-ALL). Despite continual improvements in treatment over many years, ALL is still associated with a significant mortality, and relapsed ALL continues to contribute greatly to the overall morbidity and mortality of childhood cancer. Several chromosomal abnormalities are specifically associated with T-ALL. The most common recurring breakpoints are within the 14q11, 7q32-q36 and 7p15 bands, which contain the T-cell receptor genes *TCRA/D*, *TCRB* and *TCRG*, respectively, and they are involved in 30–35% of T-ALL cases.^{1–4}

Molecular analyses of the chromosomal breakpoints have identified several T-cell oncogenes, and most of them have been formally shown to be tumorigenic.⁵ Remarkably, the majority of T-cell oncogenes belong to a number of classic transcription factor families whose expression is most often intended for lineages other than T cells. The factors deregulated in T-ALL comprise the *HOX11* homeobox genes (see below), *LMO1* and *LMO2* which contain duplicated LIM zinc-finger motifs, and *MYC*, *TAL1* (*SCL*), *TAL2* and *LYL1* which all encode helix–loop–helix proteins. A recent study on gene expression profiles in T-ALL confirmed the activation of these transcription factors to be a hallmark in these leukaemias.⁶

The *HOX11* and the closely related *HOX11L2* genes were both identified at recurrent chromosomal breakpoints in T-ALLs.^{7–13} The cryptic chromosomal translocation t(5;14)(q35;q32) has been reported to be present in approximately 20% of T-ALL cases,^{12,13} although a recent report suggests that the frequency may be even higher.¹⁴ Translocations leading to *HOX11* gene activation are present in 4–7% of T-ALLs, either by t(10;14)(q24;q11) or t(7;10)(q35;q24).^{4,15} However, work in our laboratory suggested that a larger proportion of T-ALLs exhibit deregulated *HOX11* expression when molecularly detectable abnormalities are included,¹⁶ and this was recently confirmed.⁶

At the time of diagnosis of ALL in children, several clinical and cytogenetic features are of prognostic significance. The assessment is primarily based on age and white blood cell (WBC) count; however, early response to therapy has emerged as an important prognostic variable.¹⁷ In B-lineage ALL, cytogenetic abnormalities have strong prognostic associations, contrasting with paediatric T-ALL, where cytogenetic features have little or no predictive value.¹⁵ However, one study reported better survival for patients with normal karyotypes and with t(10;14).⁴ Therefore, we have examined the prognostic significance of *HOX11* expression in T-ALL patients. In our previous study on a small group of patients, *HOX11* expression was measured by conventional polymerase chain reaction (PCR). The much larger group of T-ALL patients studied here allowed us to examine this question further and to assess corresponding cytogenetic aberrations at 10q24 and clinical outcome. In addition, the use of multiplex real-time quantitative reverse-transcriptase PCR (QRT-PCR) achieved much greater accuracy and sensitivity.

Material and methods

Study patients

Bone marrow specimens were obtained from 97 children with newly diagnosed ALL, comprising 76 T-ALL and 21 B-lineage

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Received 18 June 2002; accepted 24 December 2002

Table 1 Clinical and biological presenting features of T-ALL patients

Variable	All 63 patients		30 <i>HOX11</i> -positive patients		33 <i>HOX11</i> -negative patients	
	Number	%	Number	%	Number	%
Age (years)						
1–9	36	57.1	19	63.3	17	51.5
<1 or >10	27	42.9	11	36.7	16	48.5
Sex						
Male	38	60.3	19	63.3	19	57.6
Female	25	39.7	11	36.7	14	42.4
WBC						
<50 × 10 ⁹ /l	44	69.8	20	66.7	24	72.7
>50 × 10 ⁹ /l	19	30.2	10	33.3	9	27.3
NCI ^a						
Standard risk	27	42.9	13	43.3	14	42.4
High risk	35	55.6	17	56.7	18	54.5
Infant	1	1.6	0	0.0	1	3.0
Liver						
Normal	25	39.7	10	33.3	15	45.5
Moderately enlarged	32	50.8	18	60.0	14	42.4
Markedly enlarged	6	9.5	2	6.7	4	12.1
Spleen						
Normal	21	33.3	9	30.0	12	36.4
Moderately enlarged	30	47.6	15	50.0	15	45.5
Markedly enlarged	12	19.0	6	20.0	6	18.2
Lymph nodes						
Normal	14	22.2	4	13.3	10	30.3
Moderately enlarged	31	49.2	16	53.3	15	45.5
Markedly enlarged	18	28.6	10	33.3	8	24.2
Mediastinal mass						
Normal	22	34.9	10	33.3	12	36.4
Moderately enlarged	20	31.7	11	36.7	9	27.3
Markedly enlarged	21	33.3	9	30.0	12	36.4
CNS involvement						
Not known	3	4.8	1	3.3	2	6.1
Present	4	6.3	1	3.3	3	9.1
Absent	56	88.9	28	93.3	28	84.8

^aNCI risk stratification, see Smith *et al.*²⁶

patients. They were enrolled between 1989 and 1997 on risk-adjusted treatment protocols of the Children's Cancer Group (CCG). Cases were studied based on the availability of cryopreserved Ficoll–Hypaque-enriched leukaemic blasts from bone marrow aspirates. Diagnosis was based on morphologic, biochemical and immunological features of the leukaemic cells. Immunophenotyping was performed by indirect immunofluorescence and flow cytometry using a panel of monoclonal antibodies. Cases were classified as T-lineage based on staining using antibodies to CD7 and absence of staining for CD19. The presenting features for 63 T-ALL patients are shown in Table 1.

The risk-adjusted protocols for newly diagnosed ALL patients were as follows: CCG-1882 and CCG-1961, high-risk protocols for patients age 1–9 years with WBC counts $\geq 50\,000/\mu\text{l}$ or age ≥ 10 years; CCG-1901, high-risk protocol for patients with lymphomatous features; CCG-1922 and CCG-1952, standard risk protocols for patients 1–9 years with WBC $< 50\,000/\mu\text{l}$; CCG-1953, protocol for infants with ALL. Each protocol was approved by the National Cancer Institute (NCI) and the institutional review boards of the participating CCG-affiliated institutions. Informed consent was obtained from parents, patients, or both, as deemed appropriate, according to Department of Health and Human Services guidelines.

Reference cells

Cell line PER-255^{7,8} that exhibits a t(7;10)(q35;q24) was used as a positive control for *HOX11* expression. Bone marrow specimens and T-cells purified from peripheral blood of normal individuals were used as control preparations. The latter were obtained by the E-rosetting technique¹⁸ and analysed for purity by indirect immunofluorescence and flow cytometry using a cocktail of monoclonal antibodies (CD2, CD3, CD5 and CD7).¹⁹ The analysis of the 11 T-cell preparations showed that T-cell markers were expressed on $91.3\% \pm 8.6$ of cells.

Real-time RT-PCR analysis in multiplex format

Bone marrow specimens were enriched for lymphoblasts by Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) centrifugation. Total RNA was isolated from cryopreserved specimens and the PER-255 cell line using the TRI reagent (Molecular Research Centre, Cincinnati, OH, USA) according to the manufacturer's instructions. Quantitation was performed by OD 260 nm and OD 280 nm readings on a UV spectrophotometer. For 71 specimens, generation of cDNA was carried out using Superscript II (Life Technologies, Australia) according to the manufacturer's instructions, while for the remaining specimens the

reaction was performed using AMV RT from Promega. The QRT-PCR analysis was designed to include an internal control reaction to assess whether sufficient amplifiable material was present in each sample under test. For this the β -actin gene was chosen, and we selected an amplicon with substantial differences compared to the known pseudogenes to avoid their amplification. In order to prevent efficient amplification of genomic DNA, all amplicons flanked introns. The probe for *HOX11* spans exons 1 and 2, while the β -actin probe spans exons 4 and 5. We excluded the possibility that our *HOX11* specific primers/probes could inadvertently detect *HOX11L2*, rather than the *HOX11* gene itself, by ensuring that the two 3' base pairs of the antisense extension primer both misaligned with the *HOX11L2* template and the Tm for our probe annealing to this inappropriate template was prohibitively low at <30°C. Experimental verification using *HOX11L2*-positive cells showed them to be negative when analysed using *HOX11* primers and probes.

The quality of the assay was verified by testing cDNA samples generated in the absence of reverse transcriptase. The primers for both genes were designed using PE Applied Biosystems Primer Express™ software and were supplied by Life Technologies, Australia. The primers for *HOX11* and β -actin were as follows: *HOX11* cDNA F: tccctggatggagagtaacc, *HOX11* cDNA R: cgctgcgcttctct, β -actin cDNA F: ggcaccagcacaatgaag and β -actin cDNA R: gccgatccacaggact. The probe for *HOX11* cDNA had the sequence aggacaggtcacaggtcaccctatcaga and was labelled with FAM, while the probe for β -actin cDNA, tcaagatcattgctctctctgagcgc, was labelled with VIC. The specificity of *HOX11* primers and probe was checked by Southern blot hybridisation to *HOX11* oligo. Both probes were manufactured by PE Applied Biosystems. Primers and probes were optimised for each reaction to achieve minimal threshold cycle numbers (C_T) and maximal delta Rn (fluorescence intensity over background) values. Ideally, the control reaction for the reference gene should be performed in the same tube as the test reaction, and for the measurement of *HOX11* it was possible to find conditions for a multiplex format. Hence, both reactions were performed in the same well in a final volume of 50 μ l. The final concentrations of primers and probes were as follows: *HOX11*F 200 nM, *HOX11*R 500 nM, *HOX* probe 125 nM, β -actinF 50 nM, β -actinR 25 nM and β -actin cDNA probe 100 nM. Each reaction contained 1 μ l of cDNA. The thermal cycling conditions of the ABI PRISM 7700 Sequence Detection instrument were set to 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 s at 95°C alternating with 1 min at 60°C. A calibration curve was included with each experiment using a range of concentrations of cDNA (quantitated using UV spectroscopy OD 260 nm) ranging from 0.19 to 12.25 ng/ μ l of cDNA. In order to ensure that the specimens analysed contained sufficient amplifiable material, specimens yielding a low value for β -actin ($C_T > 25$) were excluded from the analysis. The C_T value of 25 corresponds to the reading for β -actin for the lowest cDNA concentration measured in each calibration curve, and it was highly reproducible between experiments. Each specimen was analysed in duplicate, and all specimens showing discordant findings were repeated. Five bone marrow preparations from normal individuals yielded a ratio of *HOX11*/ β -actin of 0.00053 ± 0.00033 ; hence, any value greater than the mean plus 3 s.d., which is 0.0015, was scored as positive.

Cytogenetic analysis

Cytogenetic analyses were done as described previously²⁰ at local institutions on pretreatment bone marrow or peripheral

blood specimens and were centrally reviewed by at least two members of the CCG Cytogenetics Committee. Chromosome abnormalities were designated using the 1995 International System for Human Cytogenetics Nomenclature.²¹ Abnormal clones were defined as two or more metaphase cells with identical structural abnormalities or extra chromosomes, or three or more metaphase cells with identical missing chromosomes. Diagnosis of a normal karyotype required complete analysis of a minimum of 20 banded metaphases from bone marrow only.

Statistical methods

χ^2 tests were utilised to compare characteristics of patient groups according to the status of *HOX11* expression in their leukaemic blasts. The end points used for life table outcome and prognostic factor effects were event-free survival (EFS) and survival measured from time of patient entry to the study on which they were treated. EFS is defined as the time to the first occurrence of any one of the following events: induction death, nonresponse to induction therapy, relapse after initial remission at any site, death in remission and second malignant neoplasm. Life table estimates used the Kaplan–Meier (KM) method.²² Life table comparisons of outcome for the various patient subsets according to *HOX11* expression used the log-rank statistic.²³

Results

HOX11 expression in T-ALL cells from paediatric patients

Expression of *HOX11* was studied in 76 bone marrow specimens from paediatric T-ALL patients. We established a QRT-PCR method and β -actin was measured as the reference gene in a multiplex reaction. The reference gene was also used as an indicator to exclude samples that did not contain sufficient amplifiable material. The level of *HOX11* expression was determined as the ratio of *HOX11*/ β -actin and specimens were scored as detailed under Materials and methods. The 76 T-ALL specimens segregated clearly into 37 (49%) *HOX11*-expressing and 39 (51%) nonexpressing specimens. Among the positive specimens there were 15 (19.7%) specimens expressing *HOX11* at a high level (ratio ≥ 0.05) and 22 (28.9%) at a low level (ratio $> 0.0015 < 0.05$; Figure 1). The PER-255 cell line which exhibits a t(7;10)(q35;q24) and expresses *HOX11* was included for reference, showing that the expression level of this cell line was in the range of the patient specimens expressing the gene at high levels.

We previously reported *HOX11* expression to be exclusive to T-ALL since none of the 53 B-lineage ALL showed expression of the gene.¹⁶ In order to confirm this finding with the more sensitive technique used here, we included 21 ALL specimens from patients with B-lineage immunophenotype, previously investigated by the conventional PCR method.¹⁶ All of them were confirmed to be negative for *HOX11* expression (Figure 1).

In our previous studies we did not detect *HOX11* expression in normal T-cells; however, no consistent findings on this subject have been reported.^{10,24,25} In order to address the issue, we purified peripheral blood T-cells from 11 normal individuals and analysed them using the sensitive QRT-PCR method. All of them were clearly negative for *HOX11* expression (Figure 1). Taken together these findings on ALL specimens and normal T-cells demonstrate that the method to measure *HOX11* expression is

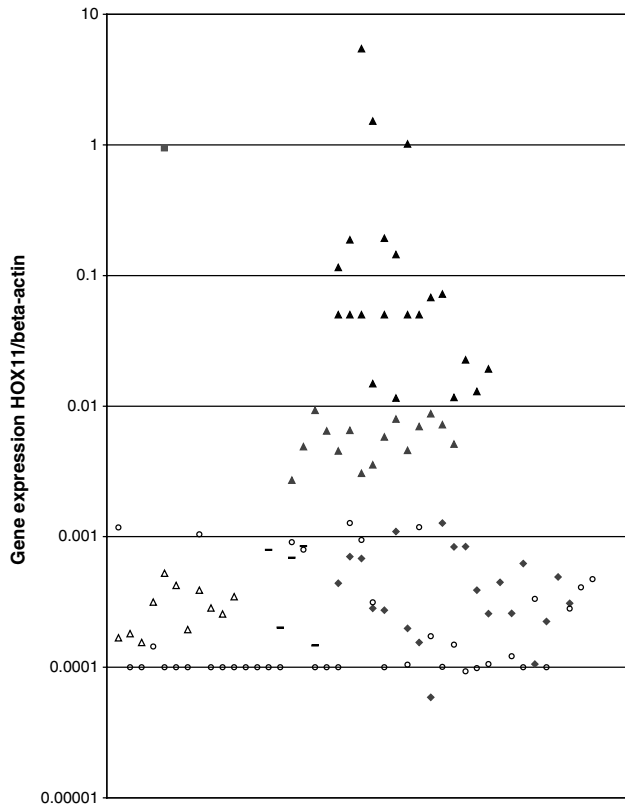


Figure 1 Gene expression of *HOX11*/ β -actin in 76 T-ALL specimens classified according to the level of expression, positive (\blacktriangle) or negative (\circ). PER-255 cell line (\blacksquare), normal bone marrow ($-$), purified peripheral T cells (\triangle) and 21 B-lineage ALL specimens (\blacklozenge). Specimens were scored based on experimental assessment of background level of the reaction, see Material and methods.

appropriate for the detection of low to high expression levels in clinical samples, since it provides sufficient sensitivity and low noise to allow unambiguous detection of signal.

Cytogenetic aberrations in T-ALL specimens expressing *HOX11*

Cytogenetic results were available for 30 of the 76 patients and they comprised the karyotypic features known to be present in T-ALL patients (Table 2). We examined the relation between *HOX11* status and cytogenetic aberrations according to the classification of normal, pseudodiploid, low hyperdiploid (47–50), high hyperdiploid (50+) or hypodiploid chromosomes. *Prima facie* there appeared to be more low hyperdiploid (47–50 chromosomes) and fewer pseudodiploid patients among the patients with leukaemia cells expressing *HOX11* compared with those showing no expression. Comparison by χ^2 tests gives a *P*-value of 0.04 for the low hyperdiploid vs nonlow hyperdiploid categories, but the comparison for pseudodiploid status is not close to a conventional significance level (*P*=0.27). Interestingly, only two of the patients showing expression of *HOX11* in their leukaemia cells had aberrations of 10q, both involving loss. One patient had monosomy 10, but had three marker chromosomes; therefore, an aberration of *HOX11* may have been present in one or more of the marker chromosomes. The second patient had deletions of both chromosome 10 homologues, del(10)(q23q25) and del(10)(q26), neither of which

Table 2 Cytogenetic results for 30 T-ALL patients

Genetic aberration	16 <i>HOX11</i> -positive patients		14 <i>HOX11</i> -negative patients	
	Number	%	Number	%
Normal	5	31	4	29
Pseudodiploid	5	29	8	57
Hyperdiploid 47–50	5	29	0	0
Hyperdiploid >50	1	6	1	7
Hypodiploid	1	6	1	7
Abnormal 10q24	2	12	0	0
TCR breakpoint	4	24	2	14
Abnormal 9p	2	12	3	21
Philadelphia chromosome	0	0	1	7
del(6p)	1	6	3	21
>1 abnormal clone	2	12	1	7

appeared to have a breakpoint involving 10q24, the cytogenetic locus for *HOX11*.

Leukaemia cells that express *HOX11* in the absence of cytogenetically detectable aberrations at 10q24 may contain small deletions or insertions at this locus. This could be assessed by FISH technique or by a long-range PCR methodology; however, the patient material was not available for this type of analysis.

HOX11 expression and treatment outcome

We first searched for associations between *HOX11* status and clinical presentation features in 63 patients (Table 1). We failed to find correlation with any of the clinical characteristics recorded. Next, we examined treatment outcome for these patients. Figure 2 illustrates the EFS and survival analysis stratified by *HOX11* status. The 7-year EFS for the 30 patients with *HOX11*-positive leukaemia cells was 83.3% vs 75.5% for those with *HOX11*-negative cells (*P*=0.48; not shown). Similarly, the survival rates were not statistically significantly different for the two groups, 83.3% compared to 73.2% for the patients with *HOX11*-positive and *HOX11*-negative cells, respectively (*P*=0.67; not shown). We next asked whether clinical outcome is a function of the level of *HOX11* expression. Comparison of outcome for the patients showing high or low *HOX11* levels did not reach a conventional statistical significance level; however, the data revealed somewhat better outcomes for the subgroup with high *HOX11* levels in leukaemia cells compared to low level or negative for *HOX11*. The 7-year EFS rates for the *HOX11*-high group was 92.9% compared to 75.5% for the *HOX11*-negative group (*P*=0.20) and 75.0% for the *HOX11*-low group (Figure 2a). The survival rates were 92.9% for the *HOX11*-high group vs 73.2% for the negative group (*P*=0.24) and 75.0% for the *HOX11*-low group (Figure 2b). We conducted subgroup analyses for all diagnostic parameters, including WBC, age and NCI risk group stratification.²⁶ None of the groups showed a significant difference regarding treatment outcome according to *HOX11* status. We next examined clinical outcome for subgroups of patients treated on the various protocols. A total of 40 patients were treated on study protocols for high-risk patients, 20 on CCG-1901 and 20 on CCG-1961. The number of patients treated on the other study protocols were too small for statistical analysis. Interestingly, we found a different outcome according

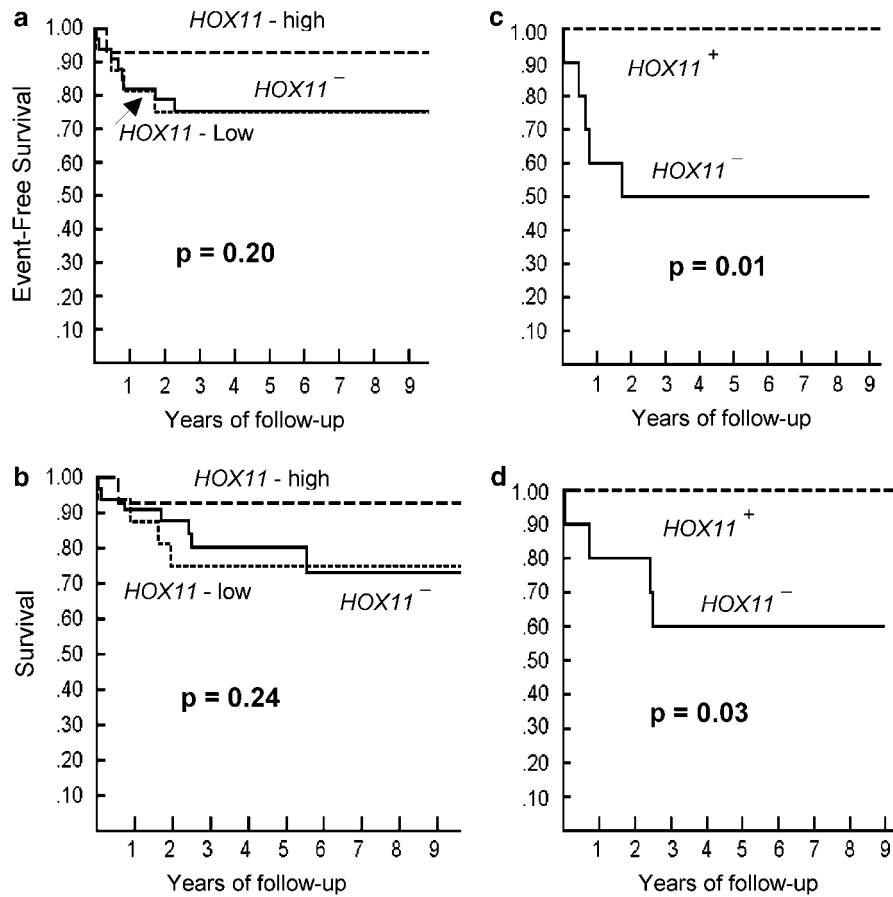


Figure 2 EFS (a) and overall survival (b) according to *HOX11* status for all 63 T-ALL patients and EFS (c) and overall survival (d) for a subgroup of 20 high-risk patients treated on CCG-1901. (a and b) Clinical outcome for patients with leukaemia cells expressing *HOX11* at high (---), at low level (-----) or negative for *HOX11* (—), (c and d) Outcome for patients with *HOX11*-positive (---) or *HOX11*-negative cells (—). *P*-values in (a) and (b) refer to comparison *HOX11* high vs *HOX11* negative.

to *HOX11* status for patients on CCG-1901 (based on New York regimen), but not for CCG-1961 (based on Berlin–Frankfurt–Münster regimen, BFM). In all, 10 (50%) of the CCG-1901 patients had *HOX11*-positive leukaemia cells, five patients showing *HOX11* expression at high level and five at low level. No EFS event occurred in this group of 10 patients, contrasting with two medullary relapses, two extramedullary relapses and one death occurring in the 10 patients with *HOX11*-negative cells. The EFS rates by year 7 were 100% for the *HOX11*-positive group vs 50% for the *HOX11*-negative group ($P=0.01$; Figure 2c) and a corresponding significant difference was found for overall survival in this group of patients as well ($P=0.03$, Figure 2d).

Discussion

Half of the T-ALL patients studied here showed *HOX11* expression in their leukaemia cells, indicating that this genetic aberration is one of the most common abnormalities in paediatric T-ALL. The recently reported aberrant expression of the *HOX11L2* gene¹² reinforces the role of homeobox oncogenes in T-ALL. The gene most frequently affected in T-ALL is *p16*, which is deleted in more than 60% of the patients.^{27,28}

The findings presented in this study revealed that deregulation of the *HOX11* gene occurred at a frequency much higher than

that reported based on cytogenetic studies. Direct cytogenetic analysis of the patient specimens showing *HOX11* expression indicated that only two of the 16 exhibited abnormalities at 10q24. These results confirm and extend our previously published findings on *HOX11*,¹⁶ and they implicate mechanisms other than chromosomal translocations involving the 10q24 locus for the deregulation of *HOX11*. Aberrant expression of several T-cell oncogenes in the absence of chromosomal abnormalities has recently been reported.⁶ Cytogenetically silent aberrations are not restricted to T-cell oncogenes, as several studies have provided evidence for similar findings involving *TEL-AML1* and *p16* in leukaemia.²⁹

There are a number of alternative mechanisms that may account for the deregulation of *HOX11*. These include subtle mutations in *cis*-regulatory sequences and translocation of enhancer elements from other genes, such as *TCR* into the *HOX11* locus. Moreover, altered methylation status of the *HOX11* promoter or *trans*-acting factors that control *HOX11* gene transcription could lead to deregulation of the gene. Our recent studies demonstrated that demethylation of the promoter accompanies reactivation of the gene.³⁰ In addition, we identified several negative elements upstream of the *HOX11* gene,³¹ but have so far failed to find evidence for their mutation in primary patient specimens. The high frequency of *HOX11* deregulation in T-ALL suggests its involvement is a key pathway for leukaemogenesis. Future molecular diagnostics may make

use of such leukaemia-specific markers as *HOX11* to detect minimal residual disease. The findings presented in this study emphasise that deregulation of *HOX11* expression is a critical step in one of the major pathways of leukaemogenesis in T-ALL. Intriguingly, the mechanisms leading to deregulation of *HOX11*, other than via translocations, are not known. Future studies will investigate the influence of chromatin configuration and possible *trans*-acting factors on *HOX11* expression.

The results in Figure 1 show that expression levels in the primary patient specimens range over several orders of magnitude, confirming the findings by Ferrando *et al.*⁶ The specimens showing the highest levels were expressing *HOX11* in the range determined for cell line PER-255 which exhibits a t(7;10)(q35;q24). For two specimens in this high-expressing group, karyotype information was available. One of them showed a deletion of chromosome 10, while the other one did not contain any abnormality affecting this chromosome. This finding demonstrates that specimens without chromosome 10 abnormalities can express the gene at a level similar to the cell line with a translocation involving the 10q24 locus. The use of the QRT-PCR technique revealed that *HOX11* expression levels vary considerably among T-ALL specimens and that those scored here as expressing *HOX11* at low levels were clearly above the control specimens. It is not known whether expression of *HOX11* at a low level produces a qualitatively different effect from expression at high level. The question whether a threshold level of *HOX11* expression may be required to exert a leukaemogenic effect warrants further investigations. Interestingly, a recently published report on mammalian *HOX* genes suggests that quantitative modulation of gene expression regulates their biological effect.³² Since transcriptional control is subject to the formation of DNA-binding complexes comprising several factors,³³ a leukaemogenic effect is presumed to be dependent not only on the level of *HOX11* expression, but also on the presence of cooperating factors.

In this study, we made use of the most accurate and sensitive methodology available to measure expression of *HOX11*, QRT-PCR. Two recent studies reported the frequency of *HOX11*-expressing cases among T-ALL patients to be between 10 and 20%,^{6,13} incidences similar to the percentage of patients determined to express *HOX11* at high level in this study. There are many differences in the method for detection of gene expression among the three studies, and they are likely to account for the apparently higher sensitivity of our method, including QRT-PCR performed as multiplex reaction and the accurate assessment of background levels in this study. Our previous investigation documented that deregulation of *HOX11* in ALL is strictly limited to T-ALL.¹⁶ Using the more sensitive method in this study, we were able to confirm that none of the specimens from B-lineage ALL patients showed expression of *HOX11*. The question whether or not *HOX11* is expressed in normal T-cells has yielded inconsistent results.^{10,24,25} To address the issue, we included 11 T-cell preparations from normal individuals and all of them were clearly negative for *HOX11* expression. Our findings demonstrated unambiguously that normal T-cells do not express *HOX11*, confirming our previous results. The fact that *HOX11* expression was not detected in normal lymphocytes excludes the possibility that the more sensitive technique simply picks up low-level background expression of the gene, rather, we have demonstrated that *HOX11* expression is clearly linked to malignancy.

The identification of patients with increased risk of treatment failure is of great importance for the management of paediatric ALL patients. Modern multiagent therapy used in CCG studies showed no difference in late EFS outcome between T-ALL and

B-lineage patients.³⁴ In a large study of 169 newly diagnosed T-ALL patients, chromosomal abnormalities, although frequently present, did not prove to be significant prognostic indicators.¹⁵ The present study showed that patients with leukaemia cells expressing *HOX11* at high level tended to have better clinical outcome compared to the other patients in the study. Interestingly, this effect was statistically significant in a subgroup of patients treated for high-risk disease on CCG-1901. Notably, this group comprised patients whose leukaemia cells expressed *HOX11* at high or at low level. In contrast, different outcome according to *HOX11* status was not observed in another group of high-risk patients who were treated on CCG-1961. The New York regimen of CCG-1901 is based on the Norton-Simon principle of utilising rotating cycles of multiple noncross resistant agents to prevent the emergence of drug resistance. In particular, an induction phase based on early intensification with high-dose alkylators and a maintenance phase of cyclic pulses of a combination of eight agents is included. CCG-1961 is based on the BFM regimen utilising a standard four-drug induction, but is unique because of the introduction of a delayed reintensification phase approximately 4 months from diagnosis at the time of minimal leukaemic burden. Lastly, the maintenance phase of CCG-1961 consists of standard continuous oral antimetabolite therapy with monthly vincristine/steroid pulses. The different findings in this study for high-risk patients treated on these two therapy protocols suggest *HOX11*-expressing leukaemia cells to be more sensitive to certain multiagent therapies compared to *HOX11*-negative cells, and that prognostic indicators may lose their relevance for patients treated on different therapeutic protocols. Prominent examples have been documented and they include T-cell phenotype and unbalanced der(19)t(1;19), which historically were associated with poor outcome in childhood ALL patients. In recent years, the introduction of more intensive therapy has improved the outcome of patients whose leukaemia cells show these features.^{34,35}

It is of interest that a large study of 343 T-ALL patients found that patients with normal karyotype or t(10;14)(q24;q11) (involving the *HOX11* locus) had a better survival rate.⁴ In agreement with the data presented here, a recent study on 58 T-ALL patients treated at St Jude Children's Research Hospital found that expression of *HOX11* in leukaemic blasts was significantly associated with favourable prognosis.⁶ Taken together, *HOX11* translocations or *HOX11* upregulation appears to be associated with better survival; however, the effect may be dependent on the therapy used. This evidence for a delicate balance between genetic profile of cancer cells and responses to particular therapeutic protocols underlines the general importance of developing more sophisticated approaches to matching molecular phenotype with treatment.

Acknowledgements

The contributing cytogeneticists were S Schonberg, S Kerman, K Rao, J Biegel, K Theil, V Murty, D Warburton, A Murch, S Schwartz, B Hurang, M Thangavelu, R Blough, L McGavran, D Roulston, H Aviv, L McMorrow, K Richkind, S Jhanwar, B Hirsch, P Cotter, N Heerema, T Glover, S Sheldon. This work was supported by the Child Health Research Foundation and the Children's Leukaemia and Cancer Research Foundation, Western Australia, the Children's Cancer Group, Arcadia, CA, USA and partly funded by NIH Grants U10-CA79726 and CA83088.

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