A subtype of childhood acute lymphoblastic leukaemia with poor treatment outcome: a genome-wide classification study

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Summary

Background Genetic subtypes of acute lymphoblastic leukaemia (ALL) are used to determine risk and treatment in children. 25% of precursor B-ALL cases are genetically unclassified and have intermediate prognosis. We aimed to use a genome-wide study to improve prognostic classification of ALL in children.

Methods We constructed a classifier based on gene expression in 190 children with newly diagnosed ALL (German Cooperative ALL [COALL] discovery cohort) by use of double-loop cross-validation and validated this in an independent cohort of 107 newly diagnosed patients (Dutch Childhood Oncology Group [DCOG] independent validation cohort). Hierarchical cluster analysis with classifying gene-probe sets revealed a new ALL subtype, the underlying genetic abnormalities of which were characterised by comparative genomic hybridisation-arrays and molecular cytogenetics.

Findings Our classifier predicted ALL subtype with a median accuracy of 90·0% (IQR 88·3–91·7) in the discovery cohort and correctly identified 94 of 107 patients (accuracy 87·9%) in the independent validation cohort. Without our classifier, 44 children in the COALL cohort and 33 children in the DCOG cohort would have been classified as B-other. However, hierarchical clustering showed that many of these genetically unclassified cases clustered with *BCR–ABL1*-positive cases: 30 (19%) of 154 children with precursor B-ALL in the COALL cohort and 14 (15%) of 92 children with precursor B-ALL in the DCOG cohort had this *BCR–ABL1*-like disease. In the COALL cohort, these patients had unfavourable outcome (5-year disease-free survival 59·5%, 95% CI 37·1–81·9) compared with patients with other precursor B-ALL (84·4%, 76·8–92·1%; p=0·012), a prognosis similar to that of patients with *BCR–ABL1*-positive ALL (51·9%, 23·1–80·6%). In the DCOG cohort, the prognosis of *BCR–ABL1*-like disease (57·1%, 31·2–83·1%) was worse than that of other precursor B-ALL (79·2%, 70·2–88·3%; p=0.026), and similar to that of *BCR–ABL1*-positive ALL (32·5%, 2·3–62·7%). 36 (82%) of the patients with *BCR–ABL1*-like disease had deletions in genes involved in B-cell development, including *IKZF1*, *TCF3*, *EBF1*, *PAX5*, and *VPREB1*; only nine (36%) of 25 patients with B-other ALL had deletions in these genes (p=0·0002). Compared with other precursor B-ALL cells, *BCR–ABL1*-like cells were 73 times more resistant to L-asparaginase (p=0·001) and 1·6 times more resistant to daunorubicin (p=0·017), but toxicity of prednisolone and vincristine did not differ.

Interpretation New treatment strategies are needed to improve outcome for this newly identified high-risk subtype of ALL.

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Introduction

Several subgroups of childhood acute lymphoblastic leukaemia (ALL) have unfavourable prognosis: T-lineage ALL (about 15% of all cases) and the precursor B-lineage subtypes with chromosomal translocations creating *MLL*-rearrangements or the *BCR–ABL1* gene fusion, each found in less than 5% of cases.[1–3] Prognostically favourable precursor B-subtypes are *TEL–AML1* (*ETV6–RUNX1*)-positive ALL (20–25% of cases), hyperdiploid ALL (>50 chromosomes; about 25% of cases), and *TCF3* (*E2A*)-rearranged ALL (often *E2A–PBX1*-positive; about 5% of cases). About 25% of patients have genetically unclassified disease (B-other);[2–4] relapses are common in these patients, indicating the need for new biological insights and treatment options for ALL.[5]

Genome-wide analyses that quantify gene expression (mRNA) in cells has provided new insights into genetic subtypes of ALL and the biological basis of drug resistance.[6,7] In two studies, patients with newly diagnosed paediatric ALL could be assigned to lineage and genetic subtypes by use of gene-expression signatures with an accuracy of more than 95%.[8,9] Once these findings are validated in independent cohorts of patients, they will provide new approaches to the classification of ALL and guide treatment decisions.

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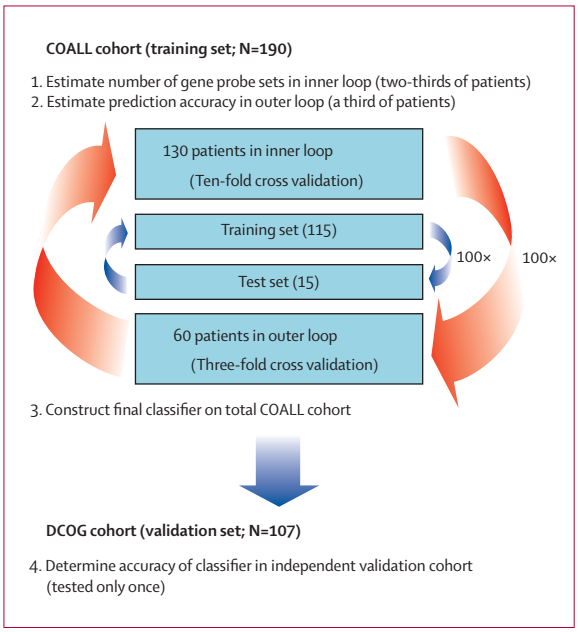

Figure 1: **Identification of a gene-expression signature enabling classification of paediatric ALL**

Panel: **Discovery of a poor prognostic subtype in paediatric acute lymphoblastic leukaemia**

1 Discovery of *BCR–ABL1*-like ALL in COALL cohort

I Visualisation of gene-expression signature in COALL cohort (190 children with ALL at initial diagnosis) by hierarchical clustering with 110 gene-probe sets previously shown to yield a high predictive accuracy in the classification of paediatric ALL (figure 1)

II Visual inspection of heat map and dendrogram: group of 30 patients without known genetic abnormalities coclustered with *BCR–ABL1*-positive cases in COALL discovery cohort (figure 2); gene-expression pattern showed most resemblance to *BCR–ABL1*-positive cases and was most distinct from other known subtypes of ALL (T-ALL, *ETV6–RUNX1*-positive, hyperdiploidy, *E2A*-rearranged, and *MLL*-rearranged)

III Outcome analysis: proportion of these 30 cases who relapse during follow-up was larger than in control group and event-free survival analysis showed that cases have a poor outcome similar to *BCR–ABL1*-positive cases—due to resemblance in gene expression pattern and outcome characteristics, this new subtype is called *BCR–ABL1*-like ALL

IV *BCR–ABL1*-like subtype comprised 19% of precursor B-ALL cases

2 Confirmation of *BCR–ABL1*-like ALL subtype in independent DCOG cohort

I Hierarchical clustering with the same 110 gene-probe sets in a second, independent group of 107 DCOG cases at initial diagnosis of ALL revealed a group of 14 cases that coclustered with *BCR-ABL1*-positive cases (figure 2); as in the COALL discovery cohort, the gene-expression pattern of these 14 cases was most similar to that of *BCR-ABL1*-positive ALL and most dissimilar from other subtypes of ALL

II Outcome analysis: *BCR-ABL1*-like cases had a poor outcome compared with control group of precursor B-ALL cases without *BCR–ABL1* fusion, which was similar to that in *BCR–ABL1*-positive ALL cases treated according to DCOG protocols

III Frequency: *BCR–ABL1*-like subtype comprised 15% of precursor B-ALL cases

3 Genetic characterisation of *BCR–ABL1*-like ALL

Comparative genomic hybridisation arrays and molecular cytogenetics were used to identify recurrent genetic abnormalities in *BCR–ABL1*-like cases: most common deletions occur in genes involved in B-cell development

Standard procedures to select gene-probe sets for classification can result in overfitting and over-interpretation of the clinical value of the gene-expression signature in diagnosis.[10] We aimed to analyse whether gene-expression signatures improve genetic classification of ALL with a double-loop cross-validation method, with further validation in an independent cohort of patients.

Methods

Patients and procedures

Bone marrow and peripheral blood samples were collected from children with newly diagnosed ALL (before initial therapy) enrolled in the German Cooperative ALL (COALL)-92/97 and Dutch Childhood Oncology Group (DCOG)-ALL-8/9 studies as approved by institutional review boards and after written informed consent was obtained. Leukaemic samples were processed as previously reported.[11] Patients' characteristics (sex, age, white blood cell count, immunophenotype, *BCR–ABL1* translocation, *MLL*-rearrangement) were recorded by COALL and DCOG study centres in Hamburg and The Hague, respectively. Cases were analysed for the presence of an *ETV6–RUNX1* fusion (fluorescence in-situ hybridisation [FISH] and RT-PCR), an *E2A*-rearrangement (split-signal FISH), *E2A–PBX1* fusion (RT-PCR), and ploidy status (DNA-index or number of chromosomes) in our laboratory.

Total RNA was extracted from samples containing more than 90% leukaemic cells with Trizol reagents (Gibco BRL, Breda, Netherlands); and RNA integrity was checked with Agilent's Bioanalyser (Santa Clara, CA, USA). cDNA and biotinylated cRNA were synthesised according to manufacturer's guidelines. The COALL cohort (n=190; 180 COALL-92/97 and ten DCOG-ALL-9 cases of the Erasmus MC-Sophia Children's Hospital) was hybridised to Affymetrix U133A GeneChips (Santa Clara, CA, USA) at St Jude Children's Research Hospital (Memphis, TN, USA).

At a later date, the DCOG cohort (107 DCOG-ALL-8 cases) was processed and hybridised to Affymetrix U133 plus 2.0 GeneChips at Erasmus MC (Rotterdam, Netherlands). Data acquisition was done with Affymetrix Microarray Analysis Suite 5.0 (COALL cohort) and GCOS 1.0 (DCOG cohort) software. The two datasets were jointly normalised with the probe sets common to both array types and the variance-stabilising normalisation procedure, which maintains the independence of both datasets.[12] All samples included had a ratio of 3′ probe to 5′ probe for β actin or glyceraldehyde-3-phosphate dehydrogenase of less than three, suggesting minimal breakdown of RNA and cRNA during the experimental procedure. The data in this paper have been deposited in NCBI's Gene Expression Omnibus[13] and are accessible through GEO Series accession numbers GSE13351 and GSE13425.

A combined double-loop cross-validation approach in a training cohort with validation of the obtained gene-expression classifier in an independent cohort was chosen

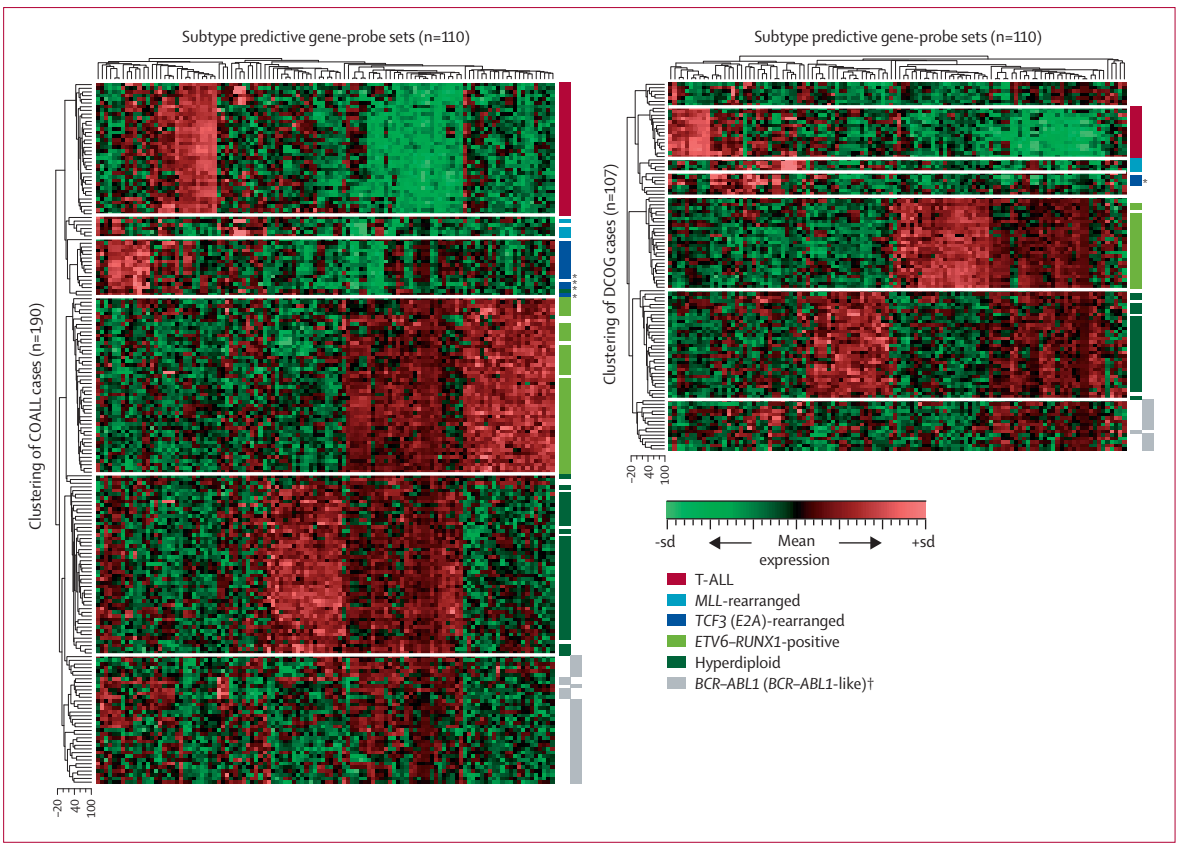

Figure 2: **Clustering of ALL subtypes by gene-expression profiles**
Hierarchical clustering of patients from the COALL (left) and DCOG (right) studies with 110 gene-probe sets selected to classify paediatric ALL. Heat map shows which gene-probe sets are overexpressed (in red) and which gene probe sets are underexpressed (in green) relative to mean expression of all gene-probe sets (see scale bar). *Patients with *E2A*-rearranged subclone (15–26% positive cells). †Right column of grey bar denotes *BCR–ABL1*-like cases.

to avoid overfitting of the data.[14] The double-loop cross-validation method used an inner loop to establish the minimum number of probe sets needed to classify ALL subtypes and an outer loop to determine the predictive value of the constructed prediction model (classifier). Figure 1 and the panel describe the classifier-building approach. In short, the inner loop was applied to two-thirds (n=130) of the COALL cohort to determine the number of probe sets needed for the prediction of six known subtypes (T-ALL, *ETV6–RUNX1*-positive, hyperdiploid, *E2A*-rearranged, *BCR–ABL1*-positive and *MLL*-rearranged ALL). The proportions of cases in each subtype were kept similar to those in the entire set of patients (webappendix). In each of 100 runs of this inner loop, patients were randomly assigned to the inner-training (90%) or the inner-test (10%) group (ten-fold cross-validation). To start, the 50 probe sets most discriminative for each subtype were selected by rank of Wilcoxon's test p value. T-ALL-associated probe sets were selected by comparison of T-ALL to precursor B-ALL cases, whereas precursor B-ALL subtype-associated probe sets were selected by comparison of the subtype to all other precursor B-ALL cases (eg, *ETV6–RUNX1*-positive *vs* non-*ETV6–RUNX1* precursor B-ALL). Because the numbers of patients with *BCR–ABL1* and MLL-rearranged subtypes were too small to give statistically significant probe sets (p>0·05; webappendix), we used the top-50 probe sets of Ross and co-workers'[9] decision-tree list as a starting set for both subtypes. Next, the least number of probe sets that best classified the patients was obtained by backwards selection starting with 300 probe sets (50 probe sets×six subtypes) with a global test that facilitates analysis of groups of probe sets, thereby lowering multiple testing errors.[15] The optimum number of probe sets determined in the inner loop served as input for a learning algorithm (ie, radial-kernal support vector machine) that enabled the construction of a prediction model (classifier) that can be used to classify single patients into subtypes. The median sensitivity of this model was estimated via three-fold cross-validation by applying the trained classifier to the remaining third (60 patients) of the COALL cohort (100 iterations; table 1). The final gene-expression classifier, trained on all 190 patients in the COALL cohort, was used to determine the prediction accuracy in the independent group of 107 patients in the DCOG cohort (table 2, figure 1).

See Online for webappendix

R (version 2.3.1) and the R packages vsn, e1071, globaltest, limma, multtest, and marray were used to run the above-mentioned analyses.[16] Probe sets that were

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)
T-lineage ALL	100 (100–100)	100 (100–100)	100 (100–100)	100 (100–100)	100 (100–100)
ETV6-RUNX1-positive	100 (100–100)	97·8 (95·7–97·8)	93·3 (87·5–93·3)	100 (100–100)	98·3 (96·7–98·3)
Hyperdiploid	100 (92·9–100)	97·8 (95·7–97·8)	92·6 (86·7–93·3)	100 (97·8–100)	96·7 (95·0–98·3)
E2A-rearranged	100 (75·0–100)	100 (98·2–100)	100 (80·0–100)	100 (98·2–100)	98·3 (98·3–100)
BCR–ABL1-positive	0 (0–0)	100 (100–100)	0 (0–0)	98·3 (98·3–98·3)	98·3 (98·3–98·3)
MLL-rearranged	0 (0–0)	100 (100–100)	0 (0–0)	98·3 (98·3–98·3)	98·3 (98·3–98·3)
Overall values	93·5 (93·5–95·7)	78·6 (78·6–85·7)	93·6 (93·2–95·6)	80·0 (76·4–84·6)	90·0 (88·3–91·7)

Data from the COALL study. Data are median (25th–75th percentile). Accuracy is for 100 iterations that include 130 cases to build the classifier and 60 other patients to determine the diagnostic test values in each interation (three-fold cross validation). Overall values based on the classification of all cases, including the B-other group.

Table 1: **Diagnostic test values for the classification of acute lymphoblastic leukaemia by three-fold cross-validation approach**

	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Accuracy
T-lineage ALL	15/15 (100%)	92/92 (100%)	15/15 (100%)	92/92 (100%)	107/107 (100%)
ETV6–RUNX1-positive	24/24 (100%)	81/83 (97·6%)	24/26 (92·3%)	81/81 (100%)	105/107 (98·1%)
Hyperdiploid	28/28 (100%)	74/79 (93·7%)	28/33 (84·8%)	74/74 (100%)	102/107 (95·3%)
E2A-rearranged	2/2 (100%)	104/105 (99·0%)	2/3 (66·7%)	104/104 (100%)	106/107 (99·1%)
BCR–ABL1-positive	0/1 (0%)	106/106 (100%)	0/0	106/107 (99·1%)	106/107 (99·1%)
MLL-rearranged	0/4 (0%)	103/103 (100%)	0/0	103/107 (96·3%)	103/107 (96·3%)
Overall values	69/74 (93·2%)	25/33 (75·8%)	69/77 (89·6%)	25/30 (83·3%)	94/107 (87·9%)

Data are number of predicted cases/total per subtype (%). DCOG cohort (107 patients) used to validate independently the predictive value of classification by gene expression signature (tested only once). Overall values based on the classification of all cases, including the B-other group. The specificity, positive predictive value, and accuracy are 100% for *E2A*-rearranged cases if the B-other case with an *E2A*-rearranged subclone (21% positive cells) is included as true positive case (webappendix).

Table 2: **Diagnostic test values for independent validation group**

	COALL cohort			DCOG cohort		
	BCR–ABL1-like (n=30)	B-other (n=119)*	p value	*BCR–ABL1*-like (n=14)	B-other (n=77)*	p value
Age at diagnosis						
Median (years)	6·0	4·5	0·14	7·4	4·0	0·15
<10 years	67% (20)	78% (93)	..	71% (10)	88% (68)	..
≥10 years	33% (10)	22% (36)	0·23	29% (4)	12% (9)	0·11
WBC at diagnosis						
Median (cells per nL)	46·3	21·0	0·004	49·0	15·6	0·010
<25 /nL	33% (10)	55% (65)	..	29% (4)	61% (47)	..
≥25 /nL	67% (20)	45% (54)	0·043	71% (10)	39% (30)	0·039
Risk group						
Low	17% (5)	44% (52)	..	..	..	..
High	83% (25)	56% (67)	0·006	..	..	..
Standard	..	..	..	7% (1)	26% (20)	..
Medium	..	..	..	50% (7)	61% (47)	..
High	..	..	..	36% (5)	9% (7)	0·032
Excluded†	..	..	..	7% (1)	4% (3)	..
Follow-up						
Continuous complete remission	63% (19)	84% (100)	..	50% (7)	78% (60)	..
Relapse	37% (11)	16% (19)	0·020	50% (7)	22% (17)	0·046

p values calculated with Mann-Whitney *U* test for continuous variables and χ^2 test for categorical variables. *Precursor B-ALL cases excluding *BCR–ABL1*-like and *BCR–ABL1*-positive cases. †Excluded because not eligible or not in treatment protocol. WBC=white blood cell count.

Table 3: **Clinical characteristics of the new *BCR-ABL1*-like subtype of ALL**

most discriminative for the six ALL subtypes were used to perform a hierarchical clustering of patients with GeneMaths 2.0 software (Applied Maths, Sint-Martens-Latem, Belgium).

To assess underlying genetic abnormalities, DNA was extracted from patients in both cohorts with Trizol reagent (Gibco BRL, Life Technologies, Breda, Netherlands) from 44 *BCR–ABL1*-like, 15 *BCR–ABL1*-positive, and 25 control precursor B-ALL (without *BCR–ABL1* translocation and non-hyperdiploid) samples containing more than 90% leukaemic cells, and stored at 4°C. 5 µg of patients' sample and 5 µg reference DNA (pooled DNA of ten male patients, Promega, Madison, WT, USA) was digested with *Alu*I (10 U) and *Rsa*I (10 U) (Invitrogen) for 2 h at 37°C and subsequently labelled overnight at 37°C with Cy-3 or Cy-5 by use of the Agilent labelling kit (Palo Alto, CA, USA). Patients' and reference DNA were pooled and mixed with 25 µg human Cot-1 DNA (Invitrogen) and blocking agent in a final volume of 250 µL hybridisation buffer (Agilent Technologies). The hybridisation mixtures were denatured at 95°C for 3 min, incubated at 37°C for 30 min, and hybridised to Agilent human genome comparative genomic hybridisation (CGH)-microarrays (105A oligoCGH-arrays containing 105 000 60-mer probes) for 42 h at 65°C. The array slides were washed in 0·5×sodium saline citrate (SSC)/0·005% Triton X-102 at room temperature for 5 min, followed by 5 min at 37°C in 0·1×SSC/0·005% Triton X-102. Slides were dried and

scanned with a 2565AA DNA microarray scanner (Agilent Technologies). Microarray images were analysed with Feature Extraction software (version 9.5; Agilent Technologies) and data were imported into array-CGH analytics software version 3.5 (Agilent Technologies). Cy5 to Cy3 and dye-swapped Cy3 to Cy5 ratios for each probe were plotted into chromosome-specific profiles. Genomic loss and gain was identified as a minimum of three adjacent probes deviating beyond the threshold of 0·8 for single copy loss and 1·8 for biallelic loss. Known large-scale copy number polymorphisms were not thought to be disease-related. Array-CGH analysis was done in duplicate for each patient to minimise false-positive results.

Bacterial artificial chromosome (BAC) clones for the detection of dic(9;20) were obtained from BAC/PAC Resource Center (Children's Hospital, Oakland, USA). BAC DNAs were isolated with DNA MiniPrep plasmid kit (Promega) and labelled with biotin-16-dUTP/digoxigenin-11-dUTP (Roche) by nick translation. A combination of chromosome 9p13.2 BAC clones (RP11-397D12 and RP11-405L18) were used in combination with 20q11.21 BAC clones (RP5-1184F4, RP5-836N17 and centromere probe). Cytospin slides were fixed in methanol, treated with RNAse and pepsin, and postfixed with formaldehyde, before being denatured for 135 s in 70% formamide and 2×SSC at 72°C. FISH probes were denatured for 8 min at 72°C and hybridised overnight at 37°C in a moist chamber. Slides were washed in 50% formamide/2×SSC and 2×SSC at 50°C, 4 min each. After dehydration through an ethanol series (70%, 85%, and 96%), they were mounted with antifade containing 4Ľ6-diamino-2-phenyl indol (DAPI) as counterstain. For each sample at least 100 interphase cells were scored. Images were taken with an epifluorescence microscope (Zeiss Axioplan 2, Sliedrecht, Netherlands) and MacProbe software (version 4.3, Applied Imaging, Newcastle upon Tyne, UK).

Cytotoxicity of prednisolone (Bufa Pharmaceutical Products, Uitgeest, Netherlands), vincristine (TEVA Pharma, Mijdrecht, Netherlands), L-asparaginase (Paronal, Christiaens, Breda, Netherlands), and daunorubicin (Cerubidine, Rhône-Poulenc Rorer, Amstelveen, Netherlands) was determined by use of a 4 day in-vitro methyl thiazol tetrazoliumbromide drug-resistance assay.[6,11] The drug concentration lethal to 50% of the leukaemic cells (LC_{50}) was used as the measure of cellular drug resistance and these values are associated with treatment outcome in children with ALL.[11,17]

The probability of disease-free survival was calculated with the Kaplan–Meier method, counting relapse as an event.[18] The standard error (SE) was determined according to the method of Peto and colleagues.[19] Cox proportional hazard analysis for univariate and multivariate analyses of potential prognostic factors.

Role of funding source

The funding sources had no role in study design, data collection, analysis, interpretation and writing or submission of the manuscript. All authors had full access to all the data in this study and final responsibility for the decision to submit for publication.

Results

Data for patients used to generate the prediction model (COALL cohort, n=190) and to validate independently the accuracy of this model for subtype classification (DCOG cohort, n=107) are summarised in the webappendix. The percentage of patients per subtype in both cohorts is

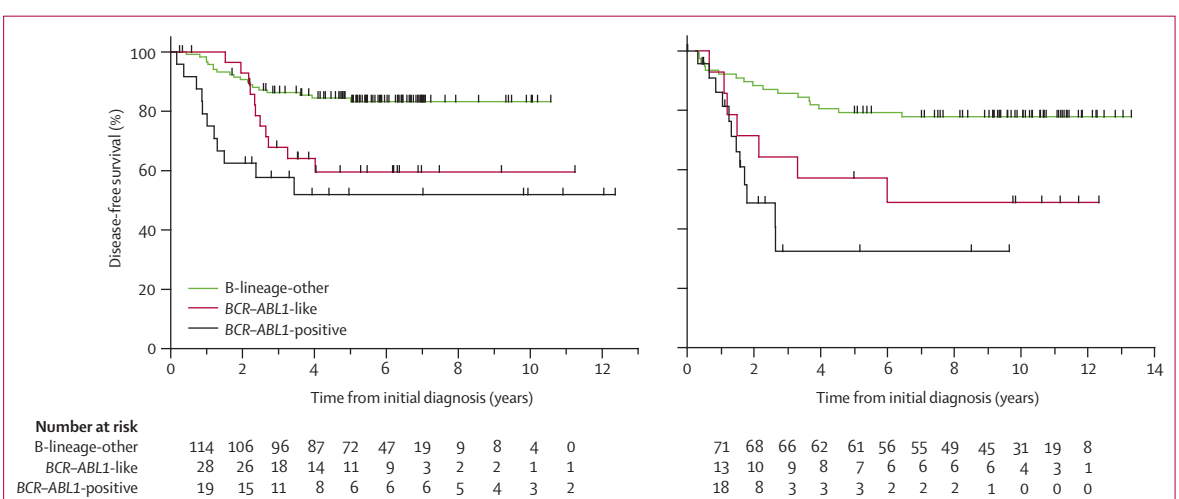

***Figure 3*: Kaplan-Meier estimates for the probability of disease-free survival (pDFS) in children with precursor B-ALL**
The COALL precursor B-ALL cohort (left) comprised 145 patients from COALL-92/97 and nine from DCOG ALL-9 treated at Sophia Children's Hospital. As reference, data for 22 patients with *BCR–ABL1*-positive disease enrolled in the COALL-92/97 protocol were included. Univariate analysis of pDFS comparing 30 *BCR–ABL1*-like with 119 remaining precursor B-ALL cases (excluding five *BCR–ABL1*-positives): p=0·012. Curves were almost the same when the nine DCOG patients were excluded. The DCOG precursor B-ALL cohort (right) comprised 92 children from DCOG-ALL8. As reference, data for 25 *BCR–ABL1*-positive cases enrolled in DCOG-ALL-7, 8, and 9 were included. Univariate analysis of pDFS comparing 14 *BCR–ABL1*-like and 77 remaining precursor B-ALL cases (excluding *BCR-ABL1*-positive case): p=0·026.

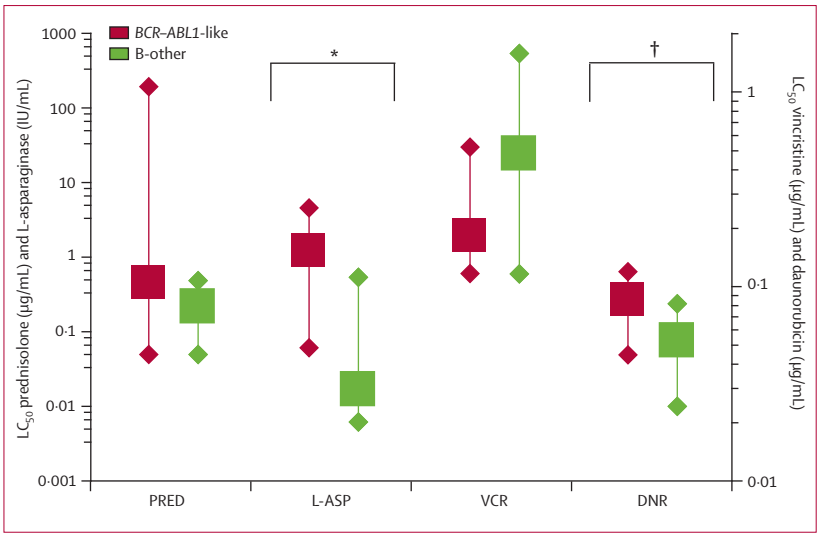

Figure 4: **In-vitro cytotoxicity of four drugs used in the treatment of paediatric ALL for *BCR–ABL1*-like and other precursor B-ALL**

Drug concentration lethal to 50% of the leukaemic cells (LC_{50}) Boxes are median LC_{50} value, bars are IQR. *BCR–ABL1*-like data are in red, those of other precursor B-ALL cases are in green. Comparison between *BCR–ABL1*-like and B-other group: p=0·001 for L-asparaginase (*) and p=0·017 for daunorubicin (†), other drugs p>0·05 (two-sided). PRED=prednisolone; L-ASP=L-asparaginase; VCR=vincristine; DNR=daunorubicin.

	BCR–ABL1-like (n=44)	*BCR–ABL1*-positive (n=15)	B-other (n=25)*
All recurrent deletions	25 (57%)	12 (80%)	5 (20%)
del(7)(p12)	6 (14%)	3 (20%)	0
del(9p)†	15 (34%)	6 (40%)	3 (12%)
del(11q22–q23)	3 (7%)	0	1 (4%)
del(13q14)	3 (7%)	0	1 (4%)
del(19p13)	4 (9%)	0	0
del(20q13)	10 (23%)	2 (13%)	1 (4%)
del(22q11)	9 (20%)	4 (27%)	2 (8%)
All recurrent amplifications	6 (14%)	3 (20%)	1 (4%)
dup(21q21–q22)	6 (14%)	3 (20%)	1 (4%)
Total‡	29 (66%)	12 (80%)	6 (24%)

All deletions or amplifications covering at least 0·5 Mbp found in at least three patients with *BCR–ABL1*-like disease. Abnormalities in the B-other group differed from both the *BCR–ABL1*-like (p=0·001) and the *BCR–ABL1*-positive (p=0·0009) groups. *Precursor B-ALL cases excluding *BCR–ABL1*-like, *BCR–ABL1*-positive and hyperdiploid cases. †Most common affected area in chromosome 9p covers 9p13.2–p21.3 region but complete loss of 9p was also reported. ‡Patients can have more than one genetic abnormality, so the total sum of cases with genetic abnormalities does not equal the sum of individual lesions.

Table 4: **Summary of recurrent genetic abnormalities found in *BCR–ABL1*-like, *BCR–ABL1*-positive, and other precursor B-ALL cases**

similar to the general distribution of cases in paediatric ALL (webappendix).[2,3]

At least 108 probe sets had the greatest sensitivity to correctly classify patients into one of six known subtypes of ALL (ie, T-ALL, *ETV6–RUNX1*-positive ALL, hyperdiploid, *E2A*-rearranged, *MLL*-rearranged, and *BCR–ABL1*-positive ALL). Each subtype was represented by 18 probe sets chosen on the basis of ranking in p values (Wilcoxon's test). Three probe sets discriminative for the *ETV6–RUNX1*-subtype had identical p values to the top 18th probe set and were therefore included. One probe set was selected for both *MLL*-rearranged and T-ALL. Hence, in total 110 probe sets, with 90 unique genes and three expressed sequence tags, were discriminative for the six ALL subtypes. Our list of probe sets only partly overlapped with those previously reported by Yeoh and co-workers[8] and Ross and colleagues[9] (ie, 26 probe sets [24%] and 31 probe sets [28%]), respectively (webappendix).[8,9]

The 110 probe sets had median sensitivity of 93·5% and median classification accuracy of 90·0% (table 1). All patients with T-ALL, *ETV6–RUNX1*-positive ALL, hyperdiploid ALL, and *E2A*-rearranged ALL were correctly identified (100% sensitivity; table 1). When applied to the independent cohort of 107 DCOG patients (tested only once), the gene-expression classifier had a sensitivity of 93·2% (identifying 69 of 74 cases) and accuracy to classify correctly both positive (six subtypes) and negative (B-other) cases of 87·9% (94 of 107; table 2).

The negative predictive value was only affected by misclassified *MLL*-rearranged and *BCR-ABL1*-positive cases in both the COALL (80·0%) and DCOG (83·3%) validation cohorts. The negative predictive value for T-ALL, *ETV6–RUNX1*-positive, hyperdiploid, and *E2A*-rearranged cases was 100% (tables 1 and 2). The specificity to classify non-*MLL* and non-*BCR–ABL1*-positive cases as negative for these translocations was 100% (tables 1 and 2 and webappendix). *MLL*-rearranged and *BCR–ABL1*-positive leukaemias clustered in distinct groups on the basis of gene-expression patterns in both cohorts of patients (figure 2). *MLL*-rearranged and *BCR–ABL1*-positive cases seem to have distinct gene-expression pattern enabling discrimination by hierarchical clustering that visualises similarities in gene-expression patterns among multiple cases, although the individual gene expression is insufficient for correct subtype prediction by the classifying model.

The positive predictive value to identify correctly patients having one of the six major subtypes was 93·6% and 89·6% in the COALL and DCOG-validation groups respectively (tables 1 and 2). Most B-other cases falsely classified as *ETV6–RUNX1*-positive, hyperdiploid, or *E2A*-rearranged could be explained in part by the presence of genetic abnormalities linking them to these subtypes (webappendix).

Hierarchical clustering with the 110 classifying gene-probe sets showed that the major subtypes of paediatric ALL separated in distinct clusters by their gene-expression signature (figure 2). The gene-expression pattern in 30 (19%) of 154 patients with precursor B-ALL—ie, 30 (68%) of 44 with B-other negative for known genetic subtypes (including *BCR–ABL1* fusion)—resembled that of the poor prognostic *BCR-ABL1*-positive disease in the COALL cohort. Analysis of the dendrogram and heatmap obtained by hierarchical clustering revealed that the gene-expression pattern of these 30 patients was most similar

to that of patients with *BCR-ABL1*-positive disease and most dissimilar from the other subtypes of ALL (figure 2). The relapse rate of these 30 patients was higher than that for those with other precursor B-ALL (37% *vs* 16%, p=0·020; table 3). These patients also had an unfavourable 5-year disease-free survival of 59·5% (95% CI 37·1–81·9) compared with 84·4% (76·8–92·1) for patients with other precursor B-ALL in the COALL cohort (p=0·012); *BCR-ABL1*-positive cases also had poor prognosis (51·9%; 23·1–80·6; figure 3). This observation prompted us to investigate further the existence of this apparent new prognostic group, which we called *BCR-ABL1*-like ALL, in the independent DCOG cohort (panel). The clustering pattern of the *BCR-ABL1*-like group was confirmed in the independent DCOG cohort, in which this group comprised 14 (15%) of 92 patients with precursor B-ALL, representing 14 (42%) of 33 patients with B-other (figure 2). Also in this DCOG validation group (being treated according to a different protocol than the COALL discovery cases), the relapse rate in patients with *BCR-ABL1*-like disease was higher than that in those with other types of precursor B-ALL (50% *vs* 22%, p=0·046; table 3). As with the COALL discovery cohort, the 5-year disease-free survival of 57·1% (31·2–83·1%) for *BCR–ABL1*-like disease was unfavourable compared with 79·2% (70·2–88·3%) in other precursor B-ALL (p=0·026; figure 3) and was like that of *BCR–ABL1*-positive disease in the DCOG cohort (32·5%, 2·3–62·7).

Age at diagnosis of patients with *BCR–ABL1*-like ALL did not differ significantly from that of patients with other precursor B-ALL types, but *BCR–ABL1*-like cases had a white-blood-cell count at diagnosis two to three times higher than B-other cases at diagnosis (table 3). Although the proportion of patients with *BCR–ABL1*-like disease with high-risk criteria is greater than that for other precursor B-ALL subtypes, 17% of patients with *BCR–ABL1*-like disease were defined as low-risk and 57% as either standard risk or medium-risk with current criteria of the COALL and DCOG protocols, respectively (table 3). The *BCR–ABL1*-like leukaemic cells were a median of 73 times (p=0·001) more resistant to L-asparaginase and 1·6 times (p=0·017) more resistant to daunorubicin than were other precursor B-ALL cases, whereas no significant difference was seen in cytotoxic effects of prednisolone and vincristine (figure 4).

Multivariate analysis including initial white blood cell count, age at diagnosis, and genetic subtypes revealed that the *BCR–ABL1*-like subtype was an independent risk factor associated with poor clinical outcome in patients receiving treatment in both COALL (hazard ratio 5·3, 95% CI 1·4–19·4; p=0·012) and DCOG cohorts (3·6, 1·1–12·1; p=0·038). These data suggest that the *BCR–ABL1*-like constitutes a large subgroup with poor prognosis that is unrecognised with current diagnostic markers.

Analysis of available karyotype data from patients with *BCR–ABL1*-like disease did not reveal a common genetic denominator (webappendix). All patients were negative for the *ETV6–RUNX1* translocation, *MLL*-rearrangement, *E2A*-rearrangement, hyperdiploidy, and *BCR–ABL1* translocation. Further genetic characterisation by array-CGH revealed seven recurrent deletions and one recurrent amplification in *BCR–ABL1*-like cases (affected region more than 0·5 million bases; table 4, figure 5). More patients with both *BCR–ABL1*-like (66%, p=0·001) and *BCR–ABL1*-positive (80%, p=0·0009) disease had recurrent abnormalities than did those with *BCR–ABL1*-negative precursor B-ALL disease (24%). Deletions in chromosome 9p and 20q were commonest, whereas recurrent amplifications were restricted to a duplication of chromosome 21q21–q22. In eight of nine patients with combined chromosome 9p and 20q deletion, the affected loci were conserved (webappendix), which might be indicative of a dicentric chromosome (dic[9;20]). The

Figure 5: **Genome-wide copy number changes in paediatric precursor B-ALL**
Genome-wide copy number data are visualised for patients with *BCR–ABL1*-like (n=44), *BCR–ABL1*-positive (n=15) and other precursor B-ALL (B-other, n=25) diseases. Deletions are visualised in red, whereas amplifications are shown in blue. Centromeres indicated by grey lines for each chromosome.

	Chromosomal location	*BCR–ABL1*-like (n=44)	*BCR–ABL1*-positive (n=15)	B-other (n=25)*
IKZF1 (*IKAROS*)	7p12.2	17 (39%)	11 (73%)	4 (16%)
TCF3 (*E2A*)	19p13.3	3 (7%)	0	0
EBF1	5q33.3	6 (14%)	0	0
PAX5	9p13.2	16 (36%)	6 (40%)	3 (12%)
VPREB1	22q11.22	15 (34%)	6 (40%)	6 (24%)
Total†	..	36 (82%)	12 (80%)	9 (36%)

No aberrations were found in other transcription factors, including *SPI1*, *BCL11A*, *E2-2*, *FOXP1*, and *LEF1*. Abnormalities in the B-other group differed from both the *BCR–ABL1*-like (p=0·0002) and the *BCR–ABL1*-positive (p=0·0098) groups. *Precursor B-ALL cases excluding *BCR–ABL1*-like, *BCR–ABL1*-positive, and hyperdiploid cases. †Patients can have more than one gene deleted, hence, the total sum of patients with deleted genes does not equal the sum of individual genes.

Table 5: **Common deletions of B-cell development genes characteristic for *BCR–ABL1*-like and *BCR–ABL1*-positive ALL**

presence of dic(9;20) was confirmed in five of six patients for whom material was available (webappendix). The *BCR–ABL1*-like cases with and without dic(9;20) did not differ in their (unfavourable) outcome (data not shown). The most common abnormality in chromosome 21 was an intrachromosomal amplification found in three of 44 patients with *BCR–ABL1*-like subtype but none of 25 control individuals. This intrachromosomal amplification is associated with a poor prognosis, and in the present study all three patients had relapses.[20] However, the low incidence of both dic(9;20) and the intrachromosomal amplification indicates that other genetic abnormalities contribute to the *BCR–ABL1*-like subtype.

Detailed analysis of areas deleted from chromosome 9p revealed that five patients had a break in the *PAX5* gene (9p13.2). Because this gene encodes a transcription factor essential to normal B-cell development, we investigated whether other genes involved in B-cell development were affected by focal deletions (less than 0·5 million bases). 36 (82%) of 44 patients with *BCR–ABL1*-like disease had one or more deletions in B-cell development genes compared with only nine of 25 (36%) with B-other ALL (p=0·0002; table 5). Genes affected included the transcription factors *IKZF1* (*IKAROS*), *TCF3* (*E2A*), *EBF1*, and *PAX5* and the pre-B-cell receptor (pre-BCR) surrogate light chain *VPREB1* (webappendix); no deletions were found in other B-cell transcription factors (*SPI1* [*PU.1*], *BCL11A*, *E2-2*, *FOXP1*, *LEF1*). In patients with true *BCR–ABL1*-positive disease, abnormalities in *IKZF1*, *PAX5*, and *VPREB1* were also common (80%, p=0·0098), which might explain the similarity in gene-expression signature between *BCR–ABL1*-like and *BCR–ABL1*-positive leukaemias.

Discussion

In this study, we used genome-wide gene-expression arrays to identify gene-probe sets that enabled classification of patients into the major subtypes of paediatric ALL. The classifier had a predictive accuracy of 90% in the discovery cohort of 190 cases with newly diagnosed ALL, and validation of this classifier in an independent cohort of 107 patients yielded a true predictive accuracy of 87·9%. Hierarchical clustering revealed that some genetically unclassified precursor B-ALL cases clustered together with *BCR–ABL1*-positive ALL cases in both the COALL discovery and DCOG validation cohort. These so-called *BCR–ABL1*-like disease had a poor prognosis, as does *BCR–ABL1*-positive disease. Genetic characterisation by comparative genomic-hybridisation arrays and molecular cytogenetics revealed that more than 80% of these cases have deletions in genes involved in B-cell development.

Several studies have reported a high classification accuracy of 90–95% for gene-expression signatures in children with newly diagnosed ALL.[8,9,21,22] However, in most studies low-abundance probe sets are eliminated by filtering procedures based on the percentage of present calls or fold-change differences, which affects the false-discovery rate of selected probe sets. Furthermore, no independent sets of patients have been used for validation of these prediction models to date.[14,23] To reduce overfitting and hence overinterpretation of the value of classification by gene-expression profiles, we used a double-loop cross-validation approach to construct a prediction classifier that was then validated in an independent group of patients. The high similarity in percentages of sensitivity, specificity, accuracy, and positive and negative predictive values between the COALL discovery and DCOG validation cohorts reassured us that our applied strategy avoids overfitting of data. Moreover, the robustness of the present classifier is emphasised by the fact that the gene-expression profiles of patients in the COALL and DCOG cohorts were generated with two different versions of the Affymetrix GeneChip and samples and arrays were processed in two different locations. All T-ALL, *ETV6–RUNX1*-positive, hyperdiploid, and *E2A*-rearranged cases were correctly classified (ie, 100% sensitivity), a finding similar to that in other studies with different strategies for probe-set selection and classifier construction.[8,9] By contrast, the sensitivity to classify *BCR–ABL1*-positive and *MLL*-rearranged cases was 0%. These small but prognostically important subtypes are always classified as B-other cases and never falsely classified as T-ALL, *ETV6–RUNX1*-positive, hyperdiploid, or *E2A*-rearranged ALL. The positive and negative predictive value of the gene-expression classifier should be high if the predicted subtype is going to be used to determine treatment. These values were 100% for T-ALL and *E2A*-rearranged ALL (including the case with an *E2A*-rearranged subclone, which was missed by routine diagnostics; tables 1 and 2 and webappendix), and hence each of these patients was correctly classified. The positive predictive value for *ETV6–RUNX1*-positive and hyperdiploid disease was not 100%, indicating that some patients are falsely assigned as *ETV6–RUNX1*-positive or hyperdiploid subtype. However, the negative predictive value for both subtypes was 100%, indicating that none of the true *ETV6–RUNX1*-positive and hyperdiploid cases was falsely assigned to another subtype

and, moreover, each predicted negative case was a true negative case. Therefore, if the gene-expression classifier is used as diagnostic tool, additional genetic verification of *ETV6–RUNX1* fusion and hyperdiploidy can be restricted to only those patients predicted to belong to these subtypes. This process reduces the number of patients screened for the *ETV6–RUNX1* fusion or ploidy by about 50%. Additional cytogenetic analysis to identify *MLL*-rearranged and *BCR–ABL1*-positive cases is only mandatory for patients with B-other ALL (only a third of patients). Before this diagnostic approach becomes widespread, prospective comparison of classification by gene expression and by conventional diagnostic techniques is needed to determine rates of clinical and technical success; this is currently being done in a Dutch nationwide setting.

Detailed studies of genes that are differentially expressed between genetic subtypes of ALL might reveal more insight into the biology of each subtype. A gene that warrants further studies is the erythropoietin receptor, which is more than seven times more highly expressed by patients with *ETV6–RUNX1*-positive disease than by those with other precursor B-ALL (webappendix) confirming other gene-expression classification studies.[8,9,24] The erythropoietin receptor might be restricted to myeloid-lineage committed progenitor cells. The increased expression in *ETV6–RUNX1*-positive ALL emphasises that this gene might have either other non-erythropoietin linked functions or that *ETV6–RUNX1*-positive cells also have myeloid characteristics. *ETV6–RUNX1*-positivity has been associated with expression of myeloid markers in paediatric ALL.[25] In cases of hyperdiploidy, one would expect that most of the selected genes are located on chromosomes often amplified in this subtype (eg, chromosomes 4, 6, 10, 14, 17, 18, 21, and X).[26] 12 of 18 selected probe sets indicative for hyperdiploidy are located on these chromosomes. However, the expression level of these genes does not reflect a gene-dose effect, because these levels of expression are more than one and a half times higher in the hyperdiploid cases than in other precursor B-ALL cases. One of the most discriminative genes associated with hyperdiploidy is the gene encoding SH3-binding protein 5 (SH3BP5). This gene, located on chromosome 3p24, is expressed almost ten times more in hyperdiploid cases than in other precursor B-ALL cases (webappendix). On the basis of the presence of specific functional domains, SH3BP5 seems to be an adaptor protein transducing signals derived from the Bruton's tyrosine kinase receptor. If functionality becomes proven, this receptor or its downstream pathway may be targeted by specific compounds, such as LFM-A13.[27]

Besides classification of disease as known subtypes with the gene-expression classifier (ie, class prediction), the same sets of gene probes could be used to discover entities with similarities in gene-expression patterns by use of hierarchical clustering (ie, class discovery). An important finding in the current study was the identification of a new ALL subtype with a gene-expression pattern resembling that of *BCR–ABL1*-positive ALL. *BCR–ABL1*-like disease affected 15–20% of patients with precursor B-ALL in both the COALL and DCOG cohorts. This newly recognised group had an unfavourable prognosis, with 5-year disease-free survival estimates of 60%, comparable to that of patients with the *BCR–ABL1*-positive ALL (figure 3). Moreover, the number of patients with *BCR–ABL1*-like disease was more than five times the number with *BCR–ABL1*-positive disease; thus, this subgroup is the most common childhood ALL with poor prognosis. By use of the current diagnostic criteria, a substantial number of patients with *BCR-ABL1*-like disease are assigned to low-risk, standard-risk, and medium-risk treatment schemes (table 3). Because *BCR-ABL1*-like cells are not more resistant to prednisolone in vitro than are other precursor B-ALL cells, patients with this subtype might not be recognised by a poor clinical response to a therapeutic window with prednisone given before combination chemotherapy, as used for risk-stratification in protocols of the International-Berlin-Frankfurt-Münster (I-BFM) study group. Previous studies indicated that both in-vitro resistance as well as a poor clinical response to prednisone and L-asparaginase are linked to an unfavourable outcome in paediatric ALL.[17,28] The present finding that *BCR–ABL1*-like cases have a poor clinical outcome and are resistant in vitro to L-asparaginase (and to a lesser extent to daunorubicin) suggest that these patients should receive more intensive therapy with available drugs or with new, more targeted drugs, although the biology of the *BCR–ABL1*-like subtype needs further study. The use of the ABL-directed tyrosine kinase inhibitor imatinib might not be indicated because *BCR–ABL1*-like leukaemia cells do not have high expression of *ABL1* mRNA. By contrast, *BCR–ABL1*-positive cases have *ABL1* mRNA expression two to four times higher than that of other precursor B-ALL cases (data not shown).

More than 80% of patients with *BCR–ABL1*-like disease have one or more abnormalities in genes involved in B-cell development. Results of a recent study by Mullighan and co-workers[29] recently suggested that *PAX5* abnormalities (mutations, deletions, and translocations) on chromosome 9p13 occur in about 30% of all patients with precursor B-ALL. The observed abnormalities in *PAX5* lowered the transcriptional activity of PAX5 protein and reduced the formation of surface IgM characteristic for more differentiated precursor B-cells.[29] By contrast with the *BCR–ABL1*-like subtype we have identified, these *PAX5* abnormalities were not associated with an unfavourable prognosis. Moreover, the deletions in the present *BCR–ABL1*-like group not only affect the *PAX5* gene locus but also commonly include larger deleted regions on chromosome 9p and deletions in other genes involved in B-cell development. This implies that the *BCR–ABL1*-like subset is different to that identified by *PAX5* abnormalities.

In summary, our study has developed and validated a new gene-expression classifier that identifies the major

subtypes of childhood ALL with a high level of accuracy and sensitivity. Importantly, we identified a new high-risk subtype with a gene-expression pattern similar to that of ALL cases containing the *BCR–ABL1* gene fusion. This *BCR–ABL1*-like subtype is characterised by abnormalities in B-cell development genes, indicating a defective (pre)B-cell receptor signalling pathway. Use of affected genes as diagnostic markers for the *BCR–ABL1*-like subtype has not been investigated. Because the new *BCR–ABL1*-like subtype is the most common poor-prognostic subtype of childhood ALL, improved treatment of this high-risk leukaemia should have a great effect on the overall cure rate of childhood ALL.

Contributors

MLDB, RXDM, WEE, and RP designed the experiment, analysed and interpretated data, and wrote the paper. MLDB and MHC did microarray experiments, MLDB and MvS did and analysed array-CGH experiments. LJCMVZ and HBB reviewed cytogenetic data. MLDB and RXDM did the statistical analysis, PJVDS provided bioinformatic resources. MLDB, STCJMP, JGCAMBG, GE, MAH, GEJS, and WAK contributed by collecting and processing of samples. All authors read, revised, and approved the paper.

Conflicts of interest

MLDB and RP have submitted a patent application (PCT/NL08/050373) for classification of leukaemia by gene-expression signatures. All other authors declared no conflict of interest.

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