STIMULUS REPORT

blood advances

Intragenic amplification of *PAX5*: a novel subgroup in B-cell precursor acute lymphoblastic leukemia?

Claire Schwab,[1,*] Karin Nebral,[2,*] Lucy Chilton,[1] Cristina Leschi,[3,4] Esmé Waanders,[5] Judith M. Boer,[6] Markéta Žaliová,[7,8] Rosemary Sutton,[9] Ingegerd Ivanov Öfverholm,[10] Kentaro Ohki,[11] Yuka Yamashita,[12] Stefanie Groeneveld-Krentz,[13] Eva Froňková,[8] Marleen Bakkus,[14] Joelle Tchinda,[15] Thayana da Conceição Barbosa,[16] Grazia Fazio,[17] Wojciech Mlynarski,[18] Agata Pastorczak,[18] Giovanni Cazzaniga,[17] Maria S. Pombo-de-Oliveira,[16] Jan Trka,[8] Renate Kirschner-Schwabe,[13,19] Toshihiko Imamura,[20] Gisela Barbany,[10] Martin Stanulla,[21] Andishe Attarbaschi,[22] Renate Panzer-Grümayer,[2] Roland P. Kuiper,[5] Monique L. den Boer,[6,23] Hélène Cavé,[3,4] Anthony V. Moorman,[1] Christine J. Harrison,[1,†] and Sabine Strehl,[2,†] on behalf of the International Berlin-Frankfurt-Münster (I-BFM) Study Group

[1]Leukaemia Research Cytogenetics Group, Wolfson Childhood Cancer Research Centre, Northern Institute for Cancer Research, Newcastle University, Newcastle upon Tyne, United Kingdom; [2]Children's Cancer Research Institute, St Anna Kinderkrebsforschung, Vienna, Austria; [3]INSERM Unité Mixte de Recherché 1131, Institut Universitaire d'Hématologie, Université Paris Diderot, Paris Sorbonne Cité, Paris, France; [4]Département de Génétique, Hôpital Robert Debré, Assistance Publique–Hôpitaux de Paris, Paris, France; [5]Department of Human Genetics, Radboud University Medical Center, Radboud Institute for Molecular Life Sciences, Nijmegen, The Netherlands; [6]Department of Pediatric Oncology, Erasmus MC–Sophia Children's Hospital, Rotterdam, The Netherlands; [7]Department of Pediatrics, University Hospital Schleswig-Holstein, Kiel, Germany; [8]Childhood Leukaemia Investigation Prague, Department of Pediatric Hematology/Oncology, 2nd Faculty of Medicine, Charles University and University Hospital Motol, Prague, Czech Republic; [9]Children's Cancer Institute, Lowy Cancer Research Centre, University of New South Wales, Sydney, Australia; [10]Clinical Genetics Section, Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden; [11]Department of Pediatric Hematology and Oncology, National Center for Child Health and Development, Tokyo, Japan; [12]Clinical Research Center, Nagoya Medical Center, National Hospital Organization, Aichi, Japan; [13]Division of Oncology and Hematology, Department of Pediatrics, Charité–Universitätsmedizin Berlin, Berlin, Germany; [14]Department of Hematology, Universitair Ziekenhuis Brussel, Vrije Universiteit Brussel, Brussels, Belgium; [15]Pediatric Oncology, Children's Research Centre, University Children's Hospital Zürich, Zürich, Switzerland; [16]Pediatric Hematology-Oncology Program, Research Center, Instituto Nacional de Câncer, Rio de Janeiro, Brazil; [17]Centro Ricerca Tettamanti, Clinica Pediatrica, Università di Milano-Bicocca, Monza, Italy; [18]Department of Pediatrics, Oncology, Hematology and Diabetology, Medical University of Lodz, Lodz, Poland; [19]German Cancer Consortium and German Cancer Research Center, Heidelberg, Germany; [20]Department of Pediatrics, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan; [21]Department of Pediatric Hematology and Oncology, Medical School Hannover, Hannover, Germany; [22]Department of Pediatric Hematology and Oncology, St Anna Children's Hospital, Medical University of Vienna, Austria; and [23]Dutch Childhood Oncology Group, The Hague, The Netherlands

Key Points

- Intragenic *PAX5* amplification defines a novel, relapse-prone subtype of B-cell precursor acute lymphoblastic leukemia with a poor outcome.

Introduction

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most common childhood malignancy, characterized by a wide spectrum of genetic abnormalities, which are used in risk stratification for treatment.[1] *PAX5* encodes a transcription factor, which plays a key role in B-cell commitment and maintenance[2] and is frequently (20% to 35%) deleted or mutated in BCP-ALL.[3-5] Germline *PAX5* mutations also occur in familial ALL.[6,7] Furthermore, chromosomal rearrangements involving *PAX5* result in the expression of potentially oncogenic *PAX5* fusion genes.[8-12] Here we present a subset of patients with BCP-ALL lacking the major cytogenetic abnormalities (*ETV6-RUNX1*, *BCR-ABL1*, and *TCF3-PBX1* fusions, high hyperdiploidy, near-haploidy, low hypodiploidy, *MLL* rearrangements, or intrachromosomal amplification of chromosome 21)[1] with intragenic amplifications of *PAX5* (*PAX5*^{AMP}).

Methods

Patients in this study originated from 15 international study groups. All participating centers obtained local ethical committee approval and written informed consent in accordance with the Declaration of Helsinki. Diagnosis of BCP-ALL was confirmed by immunophenotyping, according to standard criteria. Demographic and clinical details are summarized in supplemental Table 1.

The copy numbers of individual *PAX5* exons were determined using the SALSA multiplex ligation-dependent probe amplification (MLPA) kit P335 *IKZF1* (MRC Holland, Amsterdam, The Netherlands), as previously described (supplemental Methods).[13-15] Thirteen *PAX5*^{AMP} samples were processed on

Submitted 14 March 2017; accepted 30 May 2017. DOI 10.1182/bloodadvances.2017006734.
*C.S. and K.N. share first authorship.
†C.J.H. and S.S. contributed equally to this work.

The full-text version of this article contains a data supplement.

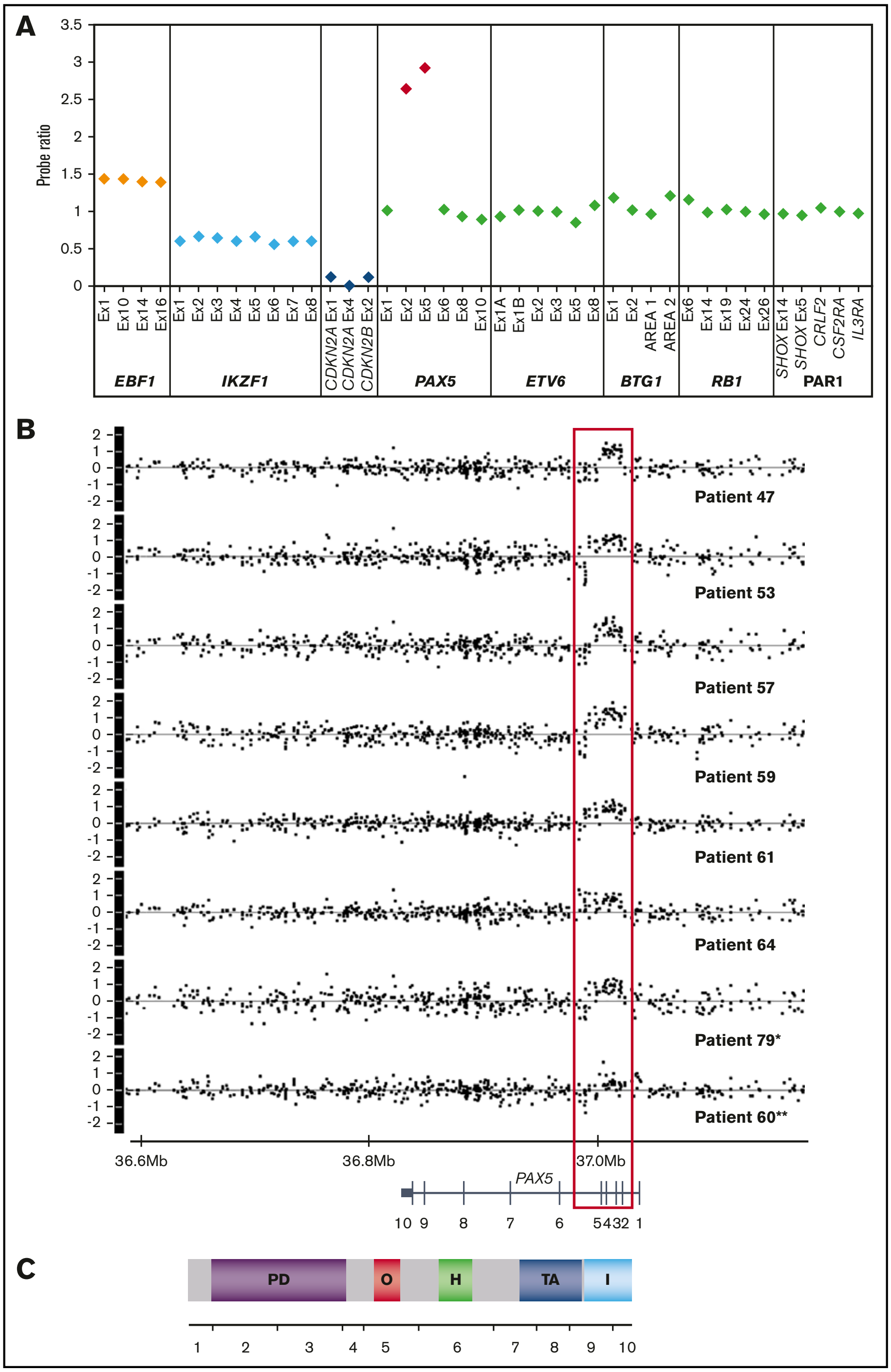

Figure 1. MLPA and SNP6.0 profiles of patients with *PAX5*^{AMP}. (A) Example of MLPA results using the P335 *IKZF1* MLPA kit. The plot shows the probe ratio for each target contained in the kit (*EBF1*, 4 probes; *IKZF1*, 8 probes; *CDKN2A/B*, 3 probes; *PAX5*, 6 probes; *ETV6*, 6 probes; *BTG1*, 4 probes; *RB1*, 5 probes; and the PAR1 region, *CRLF2*, *CSF2RA*, and *IL3RA*, 1 probe each). Probe ratio values between 0.75 and 1.3 were considered to be within the normal range, equivalent to the normal copy number of 2. Values $<$0.75 or $>$1.3 indicated loss or gain, respectively, and a value $<$0.25 indicated biallelic loss. These values correspond to copy numbers of 1, 3 and 4, and 0, respectively. A value $\geq$2.0 corresponds to a copy number of $\geq$4 and was interpreted as amplification. Approximate copy numbers of amplified exons ranged from 4 to 22 (median, 5.86). Ratio values of 1 representative patient (patient 64) showing amplification of *PAX5* encompassing exons 2 and 5, gain of *EBF1* consistent with trisomy 5 in this patient, monoallelic loss of *IKZF1*, and biallelic loss of *CDKN2A/B*. (B) Copy number profiles (log2ratio) of the *PAX5* locus of 8 patients with *PAX5*^{AMP} processed on the SNP6.0 array. *In patient 79, *PAX5* amplification was identified at relapse with no material available from diagnosis. **Patient 60 shows amplification of exon 5 only and gain of exons 1 to 4. (C) Exon and protein structure of PAX5; the amplified region encodes the DNA-binding paired domain and the octapeptid motif. PD, paired DNA-binding domain (amino acids 16-142); O, octapeptide (aa 179-186); H, homeodomain (aa 228-254); TA, transactivation domain (aa 304-391); I, inhibitory domain (aa 359-391).

SNP6.0 or CytoScan HD arrays (Affymetrix, Santa Clara, CA; supplemental Methods). Fluorescence in situ hybridization (FISH), using *PAX5* locus-specific probes, was carried out on 26 cases (supplemental Figures 1 and 2).

Survival analysis considered event-free survival, defined as time to relapse, and overall survival, defined as time to death, both censored at last contact. Very early relapse was defined as within 18 months of diagnosis, early relapse as $>$18 months and $\leq$6 months after the end of treatment, with late relapse defined as occurring $>$6 months posttreatment. Survival rates were calculated using the Kaplan-Meier method and compared using univariate Cox regression models. All analyses were performed using Intercooled Stata 14.0 (Stata, College Station, TX).

Results and discussion

PAX5^{AMP} was identified in 79 patients with BCP-ALL, at diagnosis in 77 cases; only relapse material was available from 2 patients (Figures 1 and 2A). The amplified region encompassed exons 2 and 5

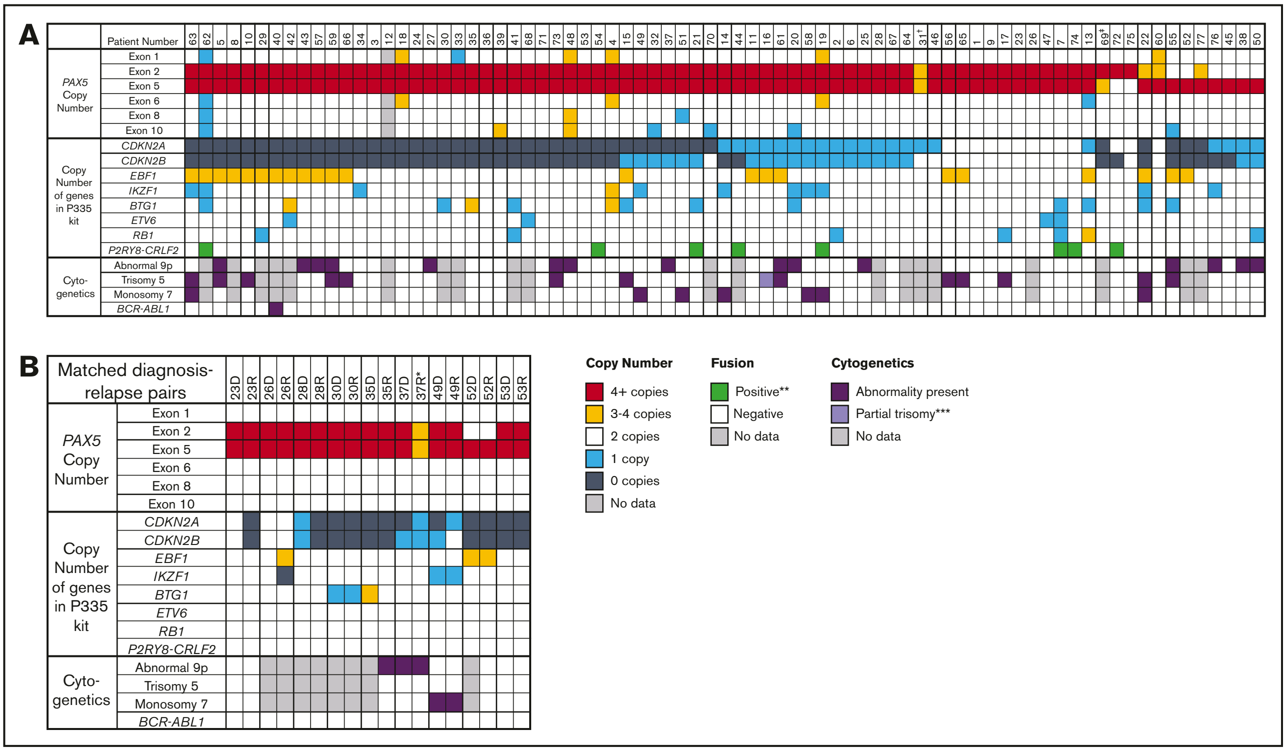

Figure 2. Genetic features of patients with *PAX5*^{AMP}. (A) Data for the 77 patients with intragenic amplification of *PAX5* identified at diagnosis. The copy numbers for each *PAX5* exon and the other genes targeted by the P335 *IKZF1* MLPA kit are shown. Cytogenetic results were available for 57 patients, with abnormalities involving the short arm of chromosome 9, trisomy 5, and monosomy 7 being the most common recurrent chromosomal abnormalities. †In patient 31, the probe ratio values for exons 2 and 5 were just below the cutoff of 2 for ≥4 copies; because the percentage of blast cells was low at 83.5%, this result was interpreted as amplification. ‡In patient 69, MLPA showed that exon 2 had a ratio of 2.42 and exon 5 of 1.69; however on the single-nucleotide polymorphism array, exons 2 to 5 were amplified. (B) Data from 9 matched diagnosis-relapse pairs. *In patient 37, the difference in copy number of the amplified exons between diagnosis and relapse was due to reduced percentage of blasts at relapse. ***P2RY8-CRLF2* fusion assessed by MLPA, FISH, and/or reverse-transcriptase polymerase chain reaction. ***Patient 16 presented with partial trisomy of chromosome 5 as a result of an unbalanced translocation involving chromosomes 1 and 5: 47,XY,der(1)(1qter-1p21::5q?34-5qter),+der(5)(5pter-5q15::1p21-1pter).

(n = 68), exon 5 only (n = 9), or exon 2 only (n = 2), encoding the DNA-binding and octapeptide domains (Figure 1). The extent of *PAX5*^{AMP} identified by MLPA was confirmed by single-nucleotide polymorphism arrays (n = 13; Figure 1B; supplemental Figure 3; GEO database accession number GSE99813). Amplification of the exons identified by MLPA was validated by quantitative genomic polymerase chain reaction (n = 18; data not shown), as previously described.[15] Because this MLPA approach did not target *PAX5* exons 3 and 4, the extent of the amplified region in cases with exon 2 or 5 amplification remains unknown. Several patients with *PAX5*^{AMP} also presented with deletions (n = 8) and gains (3-4 copies; n = 9) of other *PAX5* exons (Figure 2). The same amplification was present in 9 matched diagnosis and relapse samples (Figure 2B). FISH analysis excluded the presence of *PAX5* rearrangements and confirmed that the amplification was located within the *PAX5* locus (supplemental Figure 2). Whether *PAX5*^{AMP} results in expression of structurally mutant PAX5 proteins or loss of function remains to be determined.

Although sporadic cases with *PAX5*^{AMP} have been previously reported,[3,4,16,17] here we show *PAX5*^{AMP} to be a recurrent abnormality, occurring in 52 of 5535 patients from population-based cohorts (~1% of BCP-ALL and 3% of the B-other subgroups [33 of 1271] supplemental Table 2). The remaining 27 patients were recruited individually to this study. Apart from 1 patient case with *BCR-ABL1* fusion, *PAX5*^{AMP} was mutually exclusive of other major risk-stratifying cytogenetic markers,[1] including *IGH*, *PDGFRB/CSF1R*, *ABL1*, *ABL2*, *JAK2*, and *ZNF384* rearrangements, among 24 patient cases tested by FISH (data not shown).

Among the other genes assessed by MLPA, *CDKN2A/B* loss was the most common abnormality associated with *PAX5*^{AMP} (82%), higher than in other BCP-ALL subgroups.[3,18] Gain of *EBF1* (26%), deletion of *IKZF1* (13%), and deletion of the PAR1 region resulting in *P2RY8-CRLF2* fusion (10%) were other common alterations, suggesting a collaborative role in *PAX5*^{AMP} leukemia development.

Consistent with the MLPA data, chromosomal abnormalities involving chromosome arm 9p (26%), trisomy 5 (23%), and monosomy 7 (12%) were observed among patients with successful karyotypes (n = 57; supplemental Table 3). Notably, trisomy 5 is a rare finding in BCP-ALL in the absence of high hyperdiploidy. Our previous study of trisomy 5 as the sole cytogenetic abnormality suggested an association with poor prognosis.[19]

The main demographic and clinical features of the 77 patients with *PAX5*^{AMP} identified at diagnosis were male predominance (66%), age >10 years (25%), white blood cell count (WBC) ≥50 × 10^9 (39%), and National Cancer Institute high-risk status (55%). Minimal residual disease (MRD) data were available for 45 patients. Among ALL2003 patients with evaluable MRD (n = 8), 50% were positive at day 28 (>0.01%). Among patients treated in ALL-BFM 2000 with MRD data (n = 14), 12 were classified as MRD intermediate risk and 1 each as high and low risk, whereas all European Organisation for Research and Treatment of Cancer patients (n = 10) were intermediate risk, apart from 1 classified as high risk. From these limited data, we cannot assign an association between *PAX5*^{AMP} and MRD.

Among 74 patients with complete remission data available, 73 achieved complete remission by end of induction; 1 patient died before therapy. Relapse occurred in 40% (29 of 73) of these patients. The site of relapse, known for 22 patients, was isolated bone marrow (n = 16), extramedullary (n = 3), or combined relapse (n = 3). The time to relapse (median, 2.1 years) was known for 25 patients, with a ratio of very early to early to late relapse of 9:10:6, classifying 15 (55%) as high risk according to current criteria.[20] Among patients experiencing relapse with sufficiently long follow-up, 17 (59%) died (relapse, n = 9; infection in remission, n = 3; unknown, n = 5), and 10 remained alive >3 years postrelapse.

The 5-year EFS and OS rates, evaluable for 74 patients, were 49% (95% confidence interval [CI], 36%-61%) and 67% (95% CI, 54%-77%), respectively. To identify risk factors, we examined the effects of age, WBC, National Cancer Institute status, year of diagnosis, and presence of additional genetic abnormalities, but only WBC was significant. Patients with a WBC $>50 \times 10^9$/L had a significantly increased risk of death (hazard ratio, 3.48; 95% CI, 1.46-8.32; $P = .005$). In context, these low survival rates were generated from patients diagnosed over a 22-year period (1993-2015), treated according to a wide range of trial protocols, highlighting the need for prospective studies.

The clinical, genetic, and outcome profiles of patients with *PAX5*^{AMP} were distinct from those harboring *PAX5* deletions, which occur at different incidences between BCP-ALL subgroups.[3] Although present at an increased frequency in high-risk ALL, *PAX5* deletions are not associated with an inferior outcome.[21,22] Because the number of patients with BCP-ALL with distinct *PAX5* fusions is limited, their prognostic relevance remains to be determined.

In conclusion, we have identified a rare subset of patients with BCP-ALL with *PAX5*^{AMP}, who share a distinct spectrum of genetic abnormalities, including high frequencies of *CDKN2A/B* loss and trisomy 5. A majority of these patients lack established cytogenetic abnormalities, suggesting that *PAX5*^{AMP} may define a distinct subtype of BCP-ALL. Although several patients presented with *P2RY8-CRLF2* and 1 with *BCR-ABL1*, both have been reported as secondary changes occurring alongside primary genetic abnormalities.[3,23,24] Where matched diagnosis and relapse samples were available, the same amplification was present at both time points, indicating that *PAX5*^{AMP} may be an important driver of leukemogenesis. Because patients with *PAX5*^{AMP} showed a high incidence of relapse, we recommend testing for *PAX5*^{AMP} in future ALL trials to determine its true prognostic impact.

Acknowledgments

This work was supported by Bloodwise (formerly Leukaemia and Lymphoma Research) and the Anniversary Fund of the Austrian National Bank (OeNB grant 14133) (K.N.). M.Z., E.F., and J. Trka are supported by NV15-30626A and 00064203 (FN Motol). E.W. is supported by the Dutch Cancer Society (KUN2012-5366). R.S. is supported by National Health and Medical Research Council Australia (APP1057746). M.L.d.B. and J.M.B. are supported by the KiKa Foundation (Kinderen Kankervrij) and the European Union's Seventh Framework Program (FP7/2007-2013) under the project European Network for Cancer Research in Children and Adolescents (ENCCA; grant agreement HEALTH-F2-2011-261474). A.P. was supported by the National Center of Research and Development (LIDER grant 031/635/l-5/13/NCBR/2014). H.C. and C.L. were supported by the European Commission Seventh Framework Programme (FP7) ERA-NET on Translational Cancer Research (TRANSCAN) project TRANSCALL. R.K.-S. is supported by the German Childhood Cancer Foundation (grant DKS 2011.14). G.C. and G.F. are supported by the Italian Association for Cancer Research (AIRC).

Authorship

Contribution: C.S., K.N., S.S., and C.J.H. designed the study; C.S., L.C., K.N., S.S., C.J.H., and A.V.M. analyzed and interpreted data; the remaining authors provided genetic and clinical data; and all authors approved the final manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

The current affiliation for E.W. and R.P.K. is Princess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands.

A list of the members of the International Berlin-Frankfurt-Münster (I-BFM) Study Group appears on the I-BFM website (https://bfminternational.wordpress.com/).

ORCID profiles: R.S., 0000-0002-0188-6005; K.O., 0000-0003-2838-4555; E.F., 0000-0002-6900-8145; J. Tchinda, 0000-0002-9450-2006; G.F., 0000-0001-7077-8422; G.C., 0000-0003-2955-4528; J. Trka, 0000-0002-9527-8608; H.C., 0000-0003-2840-1511; S.S., 0000-0002-0179-0628.

Correspondence: Sabine Strehl, Children's Cancer Research Institute, St Anna Kinderkrebsforschung, Zimmermannplatz 10, 1090 Vienna, Austria; e-mail: sabine.strehl@ccri.at; and Christine J. Harrison, Leukaemia Research Cytogenetics Group, Wolfson Childhood Cancer Research Centre, Northern Institute for Cancer Research, Newcastle University, Level 6, Herschel Building, Newcastle-upon-Tyne NE1 7RU, United Kingdom; e-mail: christine.harrison@newcastle.ac.uk.

References

1. Moorman AV. The clinical relevance of chromosomal and genomic abnormalities in B-cell precursor acute lymphoblastic leukaemia. *Blood Rev*. 2012;26(3):123-135.
2. Medvedovic J, Ebert A, Tagoh H, Busslinger M. Pax5: a master regulator of B cell development and leukemogenesis. *Adv Immunol*. 2011;111:179-206.
3. Schwab CJ, Chilton L, Morrison H, et al. Genes commonly deleted in childhood B-cell precursor acute lymphoblastic leukemia: association with cytogenetics and clinical features. *Haematologica*. 2013;98(7):1081-1088.

4. Mullighan CG, Goorha S, Radtke I, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature*. 2007;446(7137):758-764.

5. Den Boer ML, van Slegtenhorst M, De Menezes RX, et al. A subtype of childhood acute lymphoblastic leukaemia with poor treatment outcome: a genome-wide classification study. *Lancet Oncol*. 2009;10(2):125-134.

6. Shah S, Schrader KA, Waanders E, et al. A recurrent germline PAX5 mutation confers susceptibility to pre-B cell acute lymphoblastic leukemia. *Nat Genet*. 2013;45(10):1226-1231.

7. Auer F, Rüschendorf F, Gombert M, et al. Inherited susceptibility to pre B-ALL caused by germline transmission of PAX5 c.547G>A. *Leukemia*. 2014;28(5):1136-1138.

8. Coyaud E, Struski S, Prade N, et al. Wide diversity of PAX5 alterations in B-ALL: a Groupe Francophone de Cytogenetique Hematologique study. *Blood*. 2010;115(15):3089-3097.

9. Nebral K, Denk D, Attarbaschi A, et al. Incidence and diversity of PAX5 fusion genes in childhood acute lymphoblastic leukemia. *Leukemia*. 2009;23(1):134-143.

10. Kurahashi S, Hayakawa F, Miyata Y, et al. PAX5-PML acts as a dual dominant-negative form of both PAX5 and PML. *Oncogene*. 2011;30(15):1822-1830.

11. Fortschegger K, Anderl S, Denk D, Strehl S. Functional heterogeneity of PAX5 chimeras reveals insight for leukemia development. *Mol Cancer Res*. 2014;12(4):595-606.

12. Fazio G, Cazzaniga V, Palmi C, et al. PAX5/ETV6 alters the gene expression profile of precursor B cells with opposite dominant effect on endogenous PAX5. *Leukemia*. 2013;27(4):992-995.

13. Krentz S, Hof J, Mendioroz A, et al. Prognostic value of genetic alterations in children with first bone marrow relapse of childhood B-cell precursor acute lymphoblastic leukemia. *Leukemia*. 2013;27(2):295-304.

14. van der Veer A, Waanders E, Pieters R, et al. Independent prognostic value of BCR-ABL1-like signature and IKZF1 deletion, but not high CRLF2 expression, in children with B-cell precursor ALL. *Blood*. 2013;122(15):2622-2629.

15. Schwab CJ, Jones LR, Morrison H, et al. Evaluation of multiplex ligation-dependent probe amplification as a method for the detection of copy number abnormalities in B-cell precursor acute lymphoblastic leukemia. *Genes Chromosomes Cancer*. 2010;49(12):1104-1113.

16. Familiades J, Bousquet M, Lafage-Pochitaloff M, et al. PAX5 mutations occur frequently in adult B-cell progenitor acute lymphoblastic leukemia and PAX5 haploinsufficiency is associated with BCR-ABL1 and TCF3-PBX1 fusion genes: a GRAALL study. *Leukemia*. 2009;23(11):1989-1998.

17. Ofverholm I, Tran AN, Heyman M, et al. Impact of IKZF1 deletions and PAX5 amplifications in pediatric B-cell precursor ALL treated according to NOPHO protocols. *Leukemia*. 2013;27(9):1936-1939.

18. Sulong S, Moorman AV, Irving JA, et al. A comprehensive analysis of the CDKN2A gene in childhood acute lymphoblastic leukemia reveals genomic deletion, copy number neutral loss of heterozygosity, and association with specific cytogenetic subgroups. *Blood*. 2009;113(1):100-107.

19. Harris RL, Harrison CJ, Martineau M, Taylor KE, Moorman AV. Is trisomy 5 a distinct cytogenetic subgroup in acute lymphoblastic leukemia? *Cancer Genet Cytogenet*. 2004;148(2):159-162.

20. Bhojwani D, Pui CH. Relapsed childhood acute lymphoblastic leukaemia. *Lancet Oncol*. 2013;14(6):e205-e217.

21. Moorman AV, Enshaei A, Schwab C, et al. A novel integrated cytogenetic and genomic classification refines risk stratification in pediatric acute lymphoblastic leukemia. *Blood*. 2014;124(9):1434-1444.

22. Mullighan CG, Su X, Zhang J, et al; Children's Oncology Group. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. *N Engl J Med*. 2009;360(5):470-480.

23. Dun KA, Vanhaeften R, Batt TJ, Riley LA, Diano G, Williamson J. BCR-ABL1 gene rearrangement as a subclonal change in ETV6-RUNX1–positive B-cell acute lymphoblastic leukemia. *Blood Adv*. 2016;1(2):132-138.

24. Morak M, Attarbaschi A, Fischer S, et al. Small sizes and indolent evolutionary dynamics challenge the potential role of P2RY8-CRLF2-harboring clones as main relapse-driving force in childhood ALL. *Blood*. 2012;120(26):5134-5142.