STIMULUS REPORT

blood advances

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

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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

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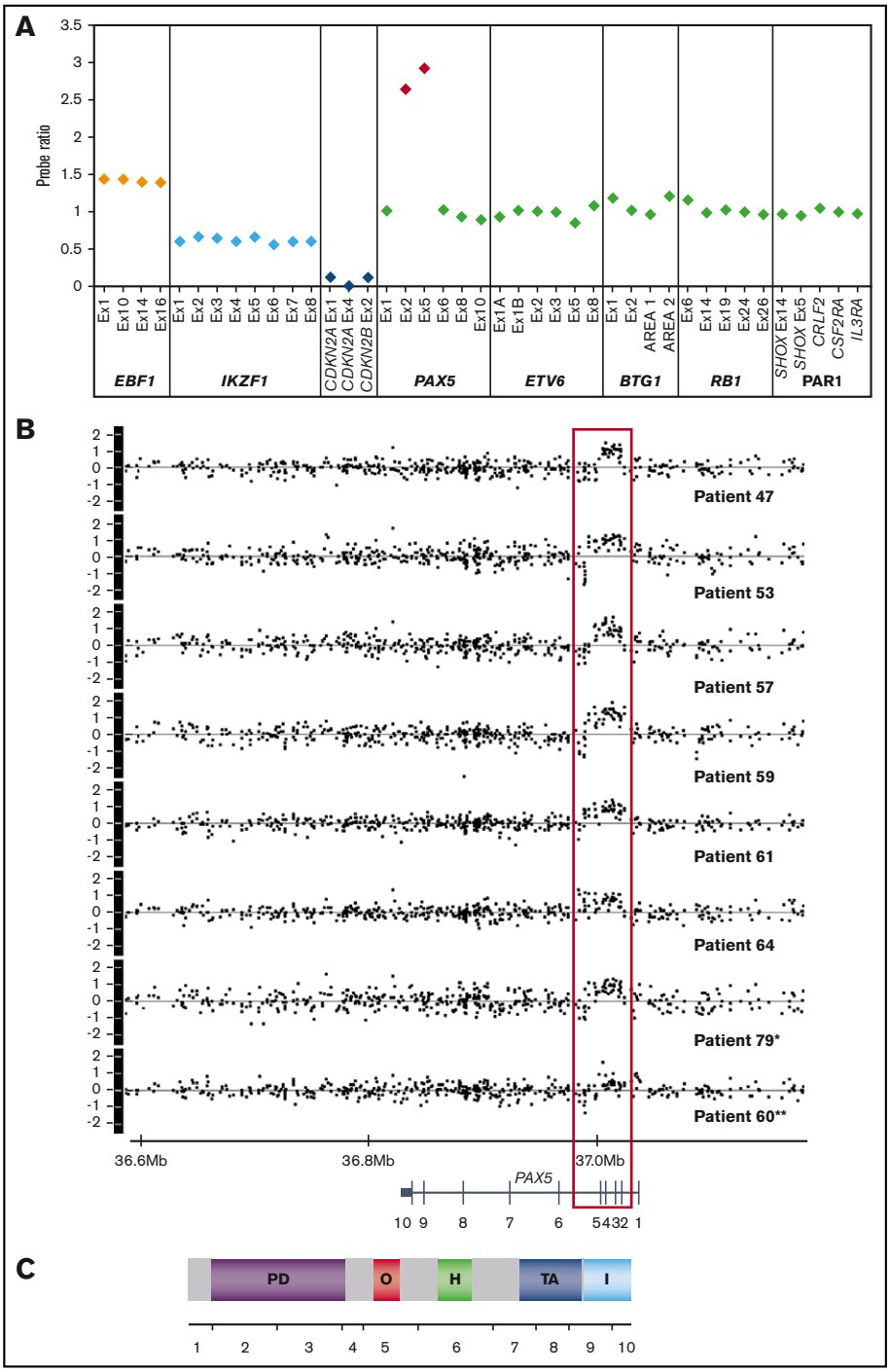

Figure 1. MLPA and SNP6.0 profiles of patients with *PAX5*^{AMP}. (A) Example of MLPA results using the P335 *IKZF1* MLPA kit. The plot shows the probe ratio for each target contained in the kit (*EBF1*, 4 probes; *IKZF1*, 8 probes; *CDKN2A/B*, 3 probes; *PAX5*, 6 probes; *ETV6*, 6 probes; *BTG1*, 4 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 ≥ 2.0 corresponds to a copy number of ≥ 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 ≤ 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

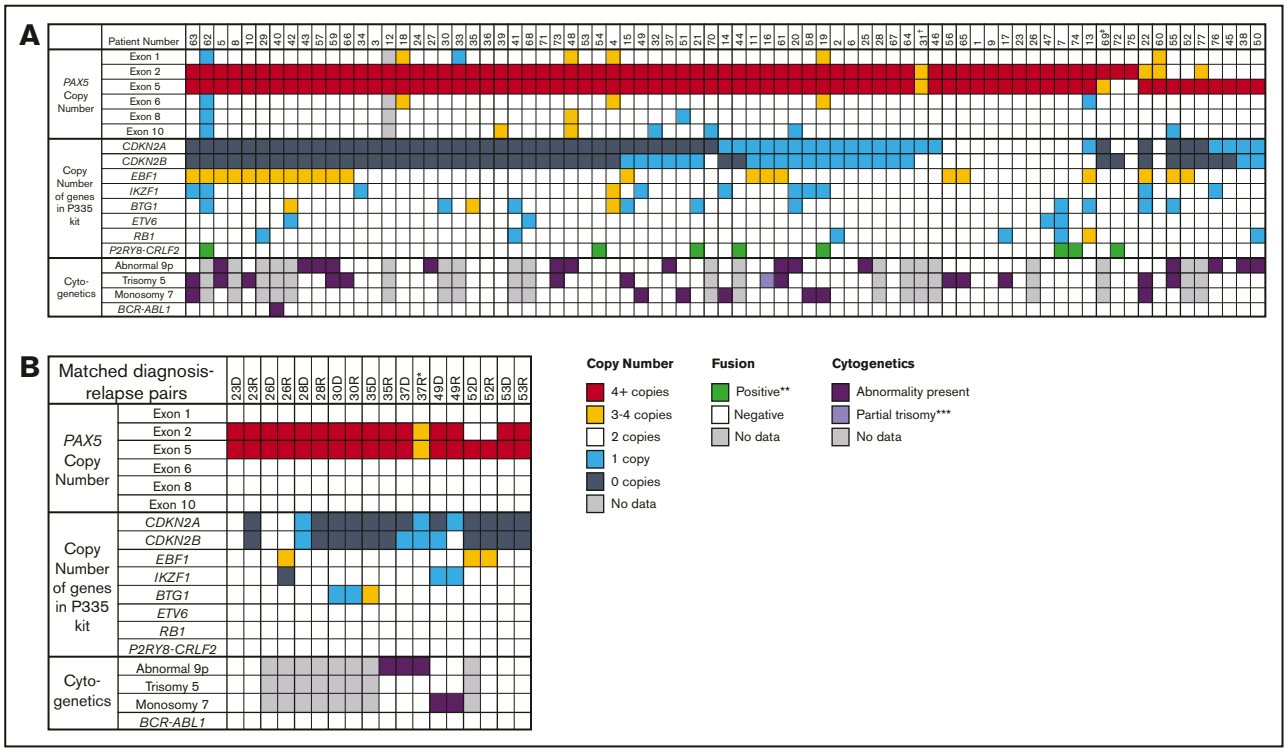

Figure 2. Genetic features of patients with *PAX5*^{AMP}. (A) Data for the 77 patients with intragenic amplification of *PAX5* identified at diagnosis. The copy numbers for each *PAX5* exon and the other genes targeted by the P335 *IKZF1* MLPA kit are shown. Cytogenetic results were available for 57 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.

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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.

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