Frequency and prognostic impact of PAX5 p.P80R in pediatric acute lymphoblastic leukemia patients treated on an AIEOP-BFM acute lymphoblastic leukemia protocol

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Abstract
PAX5 is a member of the paired box (PAX) family of transcription factors involved in B-cell development. PAX5P80R has recently been described as a distinct genetic B-cell precursor (BCP) acute lymphoblastic leukemia (ALL) subtype with a favorable prognosis in adults. In contrast, an unfavorable outcome has been observed in children. Our aim was to determine the frequency of PAX5P80R in childhood BCP-ALL treated according to the Associazione Italiana Ematologia ed Oncologia Pediatrica-Berlin-Frankfurt-Muenster (AIEOP-BFM) ALL 2000 protocol and to evaluate its clinical significance within this study cohort. The analyses included 1237 patients with ALL treated in the AIEOP-BFM ALL 2000 trial with complete information for copy number variations (CNVs) of IKZF1, PAX5, ETV6, RB1, BTG1, EBF1, CDKN2A, CDKN2B, and ERG. A customized TaqMan genotyping assay was used to screen for PAX5P80R. Sanger sequencing was used to confirm PAX5P80R-positive results as well as to screen for second variants in PAX5. Agilent CGH + SNP arrays (e-Array design 85 320; Agilent Technologies) were performed in PAX5P80R-positive patients to verify additional CNVs. Almost 2% (20/1028) of our BCP-ALL cohort were PAX5P80R-positive. White blood cell counts higher than 50 000/μl as well as male sex were significantly (P < .05) associated with PAX5P80R. Most of the PAX5P80R-positive cases were 10 years of age or older. PAX5P80R-positive samples were enriched for deletions affecting PAX5, IKZF1, CDKN2A, and CDKN2B. Compared to PAX5P80R-wildtype BCP-ALL, PAX5P80R-positive patients showed a significantly reduced 5-year overall survival (P = .042). Further studies should evaluate the interaction of PAX5P80R with other genetic aberrations to further stratify intermediate risk pediatric BCP-ALL.

Keywords
acute lymphoblastic leukemia, AIEOP-BFM ALL, B-cell precursor ALL, PAX5, pediatric acute lymphoblastic leukemia
1 | INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common malignancy of childhood and adolescence. Improvement of therapeutic strategies, including application of risk-adapted treatment employing prognostic markers such as minimal residual disease (MRD), has increased long-term survival rates of pediatric ALL to over 80%. In addition to treatment response a broad spectrum of genetic aberrations is increasingly being used for patient stratification. High-resolution genome- and transcriptome-wide analyses have led to the description of new biological subgroups and advanced treatment strategies in pediatric ALL. Improved definitions of alterations or signatures associated with a high relapse risk may help to further improve patients’ outcome. Potential targets for therapy de-escalation might be able to reduce late therapeutic effects.

One of the genes most commonly affected by deletions, amplifications, translocations, or point mutations in ALL is PAX5. A member of the paired box (PAX) family of transcription factor genes and encodes a B-cell lineage-specific activator protein, which controls the identity of B lymphocytes throughout B-cell development from the pro-B to the mature B-cell. A germline PAX5 variant conferring susceptibility to pre-B-cell ALL has lately been described and intragenic amplification of PAX5 has been shown to be related to poor outcome and relapses. A nucleotide exchange at aminoacid codon 80 (c.239C > G; NM_016734.2) of the PAX5 gene (PAX5) causes a proline to arginine exchange in the DNA-binding domain of the PAX5 protein (Figure 1A). This somatic PAX5 variant has been observed with a frequency of 0.4% in standard ALL and 3.1% in high-risk pediatric B-cell precursor (BCP) ALL. Importantly, in nearly all cases a biallelic alteration has been observed. Due to its accumulation in the intermediate to poor outcome group, we aimed to investigate if PAX5 biallelic alteration represents a prognostic marker in pediatric ALL as observed in adult ALL.

2 | MATERIALS AND METHODS

The present study included 1237 ALL patients diagnosed between August 1999 and May 2009 who have been enrolled in the international multicenter trial Associazione Italiana Ematologia ed Oncologia Pediatrica-Berlin-Frankfurt-Muenster ALL 2000 on treatment of pediatric ALL in Germany and had complete information available for copy number alterations of IKZF1, PAX5, ETV6, RB1, BTG1, EBF1, CDKN2A, CDKN2B, Xp22.33/Yp11.31 (PAR1 region; CRLF2, CSF2RA, and IL3RA genes), and ERG. In addition, included patients had to have diagnostic DNA available for further molecular analyses (1237 out of 1232). A customized TaqMan genotyping assay (PAX5_P80R_ANH6G97; ThermoFisher) was used to screen for PAX5 and positive results were confirmed by Sanger sequencing (Supplementary Table S1).

3 | RESULTS

We identified 20 PAX5-positive cases (1.9% of 1028 BCP-ALL, Supplementary Figure S1). As expected, none of the 197 patients with T-ALL were PAX5-positive (and none of the 12 patients with unknown immunophenotype as well [Supplementary Figure S2]). Only B-ALL cases were considered for the following statistical analyses. For 11 of the 20 PAX5-positive patients, cytogenetic examinations were available; all of these 11 patients belonged to the B-other group; none showed BRC-ABL, T(4;11), TCF3-PBX1 or ETV6-RUNX1 fusions nor high hyperdiploidy or hypodiploidy. Yet, they were designated as B-other throughout the remaining document. For the remaining nine patients who lacked information on ETV6/RUNX1, BCR/ABL1 and MLL/AF4 no clear statement can be made regarding their classification to the B-other collective.

Next, we were interested to determine whether additional mutational events were present in the 20 PAX5-positive samples. MLPA data indicated a deletion of one PAX3 allele in nine patients, leading to loss of heterozygosity. In confirmation with an allelic loss, the TaqMan assay showed a hemizygous PAX5 genotype in eight cases (one case was addressed as heterozygous). Sequencing of the entire gene in the remaining 11 samples with no loss showed a second nucleotide variant in 10 cases. One of them appeared to be homozygous for PAX5 and nine showed a second PAX5 variant suggesting compound heterozygosity. Of those nine additional variants, five resulted in a frameshift and two were splice site variants. In addition, we identified one missense and one nonsense variant (Figure 1A). Four of the additional variants have previously been described as somatically acquired variants, the other five have not been previously described. All variants were classified as loss of function variants. In summary, only one PAX5-positive patient had no detectable second variant, whereas 19 presented a second loss of function variant leading to PAX5 biallelic alteration (and most likely complete loss of function) (Figure 1B and Supplementary Figure S2). For 18 of the PAX5-positive patients, follow-up material was collected during remission, which was used as a germline surrogate. In none of the available remission samples PAX5 was detectable, showing that PAX5 is not a germline variant but rather somatically acquired. This confirms previous observations.

MLPA data were used to further analyze the copy number profile of the PAX5-positive patients (Figure 1B). As mentioned before, nine PAX5-positive samples showed an additional PAX5 deletion due to 9p deletion including CDKN2A/B. CDKN2A was deleted biallelically in three and CDKN2B deleted biallelically in five cases. In addition, four samples had a monoallelic, intragenic IKZF1 deletion (located at 7p12.2). Two of the four IKZF1 deleted patients fulfilled the recently described IKZF1 criteria. The IKZF1 criteria are fulfilled if in addition to an IKZF1 deletion at least one additional deletion in CDKN2A and/or CDKN2B (homozygous deletion only), a deletion in PAX5 or the PAR1 region is present and an ERG deletion (ERGdel) is absent. ETV6 was deleted in one patient, as was BTG1. There were no alterations in EGR, CRLF2, EBF1, and RB1 in PAX5-positive patients.

Agilent CGH + SNP arrays (e-Array design 85 320; Agilent Technologies, Supplementary Table S2) were used to screen for additional copy number alterations in five PAX5-positive patients, who experienced an event (Figure 2A). This included the patient that appeared to be...
homozygous for \(\text{PAX5}^{\text{P80R}}\) at first (#10). Importantly, array analyses showed a 9p deletion leading to hemizygosity of \(\text{PAX5}\) in this case, too (Supplementary Figure S3). Additionally, in this case and in another one, alterations at chromosome 20 (Supplementary Figure S3) were present. All but one of the five patients had a biallelic \(\text{CDKN2A}/\text{B}\) deletion, which was in line with the MLPA data (Supplementary Table S2).

In our cohort, white blood cell counts higher than 50 000/\(\mu\)l as well as male sex were significantly \((P < .05)\) associated with the presence of \(\text{PAX5}^{\text{P80R}}\). In addition, most of the \(\text{PAX5}^{\text{P80R}}\) cases were 10 years of age or older and only 15% were MRD-negative (Supplementary Table S3). Furthermore, 70% of our \(\text{PAX5}^{\text{P80R}}\)-positive cases were initially stratified to the intermediate risk (IR) group (Figure 1B), a group of patients still positive for MRD after induction.3 IR patients neither fulfill criteria for treatment intensification, nor for therapy de-escalation and may benefit from improved treatment stratification.3

Compared to \(\text{PAX5}^{\text{P80R}}\)-wildtype BCP-ALL, \(\text{PAX5}^{\text{P80R}}\)-positive patients showed no significant differences in event-free-survival
(Figure 2A) and the cumulative incidence of relapse (Figure 2B). During the 5 years of follow-up, events were recorded for five patients: three died treatment-related, one deceased of a relapse and one relapsed but survived (Figure 2A). PAX5<sup>P80R</sup>-positive patients had a significantly poorer 5-year overall survival ($P = .042$, Figure 2C). Although the overall survival was significantly different in univariate analysis, this was not observed in multivariate analysis (Supplementary Table S4).

### DISCUSSION

PAX5<sup>P80R</sup> has been the focus of interest of several other studies. While our research was focused on children, others have examined adults or both with opposing conclusions regarding outcome. In our BCP-ALL cohort 1.9% of the patients were positive for PAX5<sup>P80R</sup>, whereas other trials have shown higher prevalence (ie, 2.4%, 5.3%, and 6.4% of BCP-ALL). Most of our PAX5<sup>P80R</sup>-positive patients were 10 years of age or older ($P < .0001$ matching the findings of increased prevalence with increasing age and in young adults. Bastian et al have described an equal sex distribution, whereas Gu et al have observed a larger proportion of males (65.9%), as we did in our cohort (75%, $P < .05$). As described previously, PAX5<sup>P80R</sup>-positive samples were enriched for deletions of PAX5, IKZF1, CDKN2A, and CDKN2B. Bastian et al have recently described PAX5<sup>Plus</sup>, a subgroup defined by these enrichments, the frequent occurrence of RAS pathway mutations and the lack of assignability to a previously known group. As formerly described none of our patients belonged to one of the known groups as well. However, we cannot confirm the favorable outcome of PAX5<sup>P80R</sup> described in adults. In fact, we observed rather intermediate to poor outcome for patients harboring PAX5<sup>P80R</sup>, which is in line with Gu et al (Figure 2).

With a rate of 1.9% in our study and the absence of established genetic risk markers, PAX5<sup>P80R</sup>-positive patients might represent a novel subgroup in BCP-ALL, especially in adolescents and young adults. This subgroup seems to be further characterized by enrichment of copy number variations (CNVs) in specific genes other than...
PAX5 and by an intermediate to poor outcome. Although PAX5P80R is not a germline variant it may represent an initiating event in the development of B-ALL, and subsequent biallelic loss of PAX5 function further contributes to neoplastic progression. One important limitation of our study is the rare occurrence of PAX5P80R in children, which resulted in a moderate case number despite the large cohort. Nevertheless, our data underline the importance of PAX5P80R in pediatric BCP-ALL and that international collaborative approaches with larger cohorts are warranted to further evaluate the interaction of PAX5P80R with other genetic aberrations (eg, additional deletions in 9p, or complex aberrations of chromosome 20) and its impact on the patient’s outcome.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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