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Impact of *IKZF1* deletions and *PAX5* amplifications in pediatric B-cell precursor ALL treated according to NOPHO protocols

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Survival rates for childhood acute lymphoblastic leukemia (ALL) have increased dramatically over the last four decades due to a combination of intensified treatment, improved supportive care and risk stratifications based on several parameters including genetic aberrations. However, 10–20% of patients treated according to modern protocols still relapse and 10% do not survive the disease.[1] Genetic lesions signaling high risk are rare and treatment failures occur across the whole spectrum of cytogenetic risk groups, while a substantial proportion of cases also lack characteristic rearrangements used for risk stratification.[2]

Deletion of the *IKZF1* locus, coding for the lymphoid transcription factor IKAROS, has recently emerged as a potential prognostic marker in B-cell precursor ALL (BCP ALL).[3] The vast majority of reported aberrations in *IKZF1* are intragenic deletions, while sequencing of the *IKZF1* gene has revealed a low frequency of point mutations.[4–7] Heterozygous deletions of the entire *IKZF1* locus or individual exons are predicted to result in haploinsufficiency, whereas deletion of a specific subset of exons (Δ4-7) results in a shorter, dominant-negative isoform, IK6.[8] Different isoforms of *IKZF1*, generated through alternative splicing, are expressed during normal B-cell development;[9] however, several studies have demonstrated that expression of the IK6 isoform is pathogenic and caused by deletion only.[10] Originally described as a characteristic of *BCR-ABL1*-positive ALL,[8] *IKZF1* deletions were later suggested to predict high relapse risk and poor prognosis in all types of BCP ALL.[3] Moreover, these deletions have been associated with a gene expression profile resembling *BCR-ABL1*-positive ALL.[11]

The aim of our study was to assess the frequency and prognostic impact of *IKZF1* mutations in a consecutive cohort of children aged 1–18 diagnosed with BCP ALL from 2001 to 2011 at the Karolinska University Hospital in Stockholm and treated according to NOPHO (Nordic Society of Paediatric Haematology and Oncology) protocols.[1] During this period 148 children were diagnosed with BCP ALL, and DNA from bone marrow aspirates obtained at diagnosis was available from 120 of those cases. The median age of the cohort was 5 years, with a male to female ratio of 1.1. The median white blood cell count at diagnosis was 9.5×10^9/l. Routine cytogenetic analysis showed that the majority of cases belonged to low-risk genetic categories (72/120). A small number showed aberrations associated with intermediate risk (13/120), and one patient was diagnosed with a high-risk, hypodiploid leukemia. In total, 34 out of 120 patients (28%) lacked risk-stratifying aberrations at diagnosis. Only two *BCR-ABL1*-positive ALL cases were diagnosed in Stockholm during the period and no DNA was available from these patients. Two of the children had Down's syndrome. After risk group assignment according to NOPHO protocols, 40% were considered standard-risk, 44% intermediate-risk and 16% high-risk ALL.

We used multiplex ligation-dependent probe amplification (MLPA) with the probe kits P335 and P202 from MRC-Holland (Amsterdam, The Netherlands) to investigate the presence of copy number alterations of *IKZF1* in DNA from bone marrow samples taken at diagnosis. All 120 cases were analyzed with the probe kit P335 that contains one probe for each exon in the *IKZF1* gene together with probes for several genes implicated in leukemia development: *IKZF1, CDKN2A/B, PAX5, EBF1, BTG1, RB1, ETV6, CRLF2, SHOX, CSF2RA* and *IL3RA*. Four samples failed MLPA due to poor sample quality. Cases that were found to harbor

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deletions in *IKZF1* were further analyzed with the high-resolution probe kit P202, which contains two probes per exon in the *IKZF1* gene, to confirm the results. The MLPA procedure was carried out according to the manufacturer's instructions (MRC-Holland). A total of 116 patients were included in the statistical analysis. All clinical data were retrieved from the NOPHO database. The SPSS software 20.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for all statistical calculations. The probabilities of event-free survival (pEFS) and overall survival (pOS) at 10 years after diagnosis were calculated using the Kaplan–Meier method, and the groups were compared using the log rank test. Cox proportional hazard regression analysis was performed to assess risk factors. In the calculation of pEFS, events were defined as induction failure, relapse, death in first remission and second malignant neoplasm. In the analysis of pOS, death from any cause was the only end point.

Deletions of *IKZF1* were detected in 16% (19/116) of the cases. Five out of nineteen (26%) harbored the partial deletion (Δ4-7) predicted to result in the dominant-negative isoform IK6. The remaining cases carried heterogeneous deletions affecting one or several exons of the *IKZF1* gene (Table 1A). All deletions were heterozygous except for one case (P4) with a homozygous deletion of the first exon of *IKZF1*.

The frequency of aberrations in other genes detected by the P335 probe kit was consistent with previous gene-dose studies of childhood ALL,[4,5] the most common being deletions of *CDKN2A/B* (41%), aberrations of *PAX5* (35%) and deletions of *ETV6* (26%). Less common were deletions of *RB1* (5.1%), *BTG1* (4.3%) and *EBF1* (1.7%). Deletion of *CSF2RA* and *IL3RA* with normal *CRLF2*, an aberration predicted to cause *CRLF2* overexpression,[12] was detected in 4.3% of cases. One of these latter cases also carried a deletion of *IKZF1* exon 4-7 (Table 1A).

One unexpected finding was a recurrent intragenic amplification of *PAX5*, which was detected in 5 out of 116 cases (4%, Table 1B). None of these five cases showed stratifying genetic markers; however, one case (P16) also had a deletion of *IKZF1*. The clinical parameters of patients with either of these aberrations are presented in Table 1. Two of the patients with *PAX5* amplification relapsed, which suggests that this aberration could represent a novel prognostic marker in the group of BCP ALL with no previously recognized stratifying genetic aberration. Intragenic amplifications of *PAX5* have previously only been reported in

Table 1. Clinical, cytogenetic and MLPA data for BCP ALL cases with *IKZF1* deletions (A) and intragenic *PAX5* amplifications (B)

(A)

Patient no.	*Age*	*Gender*	*WBC*	*Karyotype*	*RG*	*IKZF1 exons deleted*	*Other deleted genes*	*Last follow-up*
P 1	4	F	117	46,XX.ish del(9)(p21p21)x2	HR	4–7	*CDKN2A/B*	CR1
P 2	3	F	39.5	45,XX,-9,der(20)t(9;20)(q22;q11.2)	IR	4–7	*CDKN2A/B, CSF2RA, IL3RA, PAX5, ETV6*	CR1
P 3	4	F	2.5	53,XX,+9,+21,inc	SR	1–8	—	CR1
P 4	3	F	9.3	55,XX,+X,+4,+6,+10,+14,+17,+18,+21,+21[1]/ 54,XX,+X,+4,+6,+10,i(14)(q10),+17,+18,+21,+21[3]	SR	1	*PAX5, ETV6, EBF1*	CR1
P 5	1	M	20.3	46,XY.ish del(12)(p13p13),t(12;21)(p13;q22)	IR	1–8	*PAX5, ETV6, RB1*	CR1
P 6	7	M	37.9	46,XY	IR	4–7	—	CR1
P 7	7	M	6.6	48,XY,+der(1)t(1;16)(p11;?),-16,+i(21)(q10)x2	SR	4–8	*PAX5*	Dead
P 8	14	M	13.8	47,XXYc,dic(7;16)(p11;p13.2),+21c	IR	1–8	*ETV6, BTG1*	Dead
P 9	12	M	3	46,XY.ish amp(21)(q22)	IR	1–8	*RB1*	CR1
P 10	11	M	8.1	46,XY.ish t(12;21)(p13;q22)	IR	1	*CDKN2A, BTG1*	CR1
P 11	2	F	59.2	52,XX,t(3;7)(p21;p15),+8,+11,ins(12;9)(p13;p21p24),+14,+14,+21,+22	HR	2–7	*CDKN2A/B, BTG1*	CR1
P 12	15	M	26.4	52,XY,der(1)t(1;8),t(1;19),der(2)t(2;6),+der(2)t(2;6),der(4)t(4;6),-6,+der(8)t(4;8),der(9)t(6;9),+der(9)t(6;9),+der(14)t(?;14),+16,+21,+mar[a]	IR	1–5	*CDKN2A/B, PAX5, BTG1*	Dead
P 13	3	M	1.9	47,XY,+12.ish t(12;21)[3]/47,XY,idem.ish del(11)(q23q23)[3]	SR	1–3	*CDKN2A, PAX5, BTG1*	CR1
P 14	2	M	10.1	46,XY.ish del(9)(p21p21)x2	SR	4–7	*CDKN2A/B, PAX5*	CR1
P 15	6	M	65.3	48,XY,+?19,+mar inc	HR	1–8	—	CR1
P 16	11	M	60	45,XY,-4,i(8)(q1?1),der(9)t(4;9)(p?;p2?1),del(12)(p1?3),der(16)ins(16;4)(q1?2;?).ish del(9)(p21p21)	SR	2	*CDKN2A/B*	Dead
P 17	1	F	24	46,XX.ish del(12)(p13p13)t(12;21)(p13;q22)	SR	2-3	*PAX5*	CR1
P 18	4	M	1.7	57,XY,+X,+Y,+4,+6,+8,+10,+14,+17,+18,+21,+21	IR	1–8	—	CR1
P 19	15	M	285	46,XY,t(5;12)(q33;p13)	HR	4–7	—	Dead

(B)

Patient no.	*Age*	*Gender*	*WBC*	*Karyotype*	*RG*	*PAX5 exons amplified*	*Other deleted genes*	*Last follow-up*
P 16	11	M	60	45,XY,-4,i(8)(q1?1),der(9)t(4;9)(p?;p2?1),del(12)(p1?3),der(16)ins(16;4)(q1?2;?).ish del(9)(p21p21)	SR	5	*IKZF1, CDKN2A/B*	Dead
P 20	5	M	122.7	47,XY,der(4)t(X;4)(?;p16),+21c	HR	2	*CDKN2A/B, CSF2RA, IL3RA*	CR1
P 21	3	M	8.1	47,XY,+5,der(7),del(9)(p?)x2	SR	2–5	*CDKN2A/B*	CR1
P 22	3	M	28.1	46,XY,t(10;14)(p11.2-12;q11.2)	IR	2–5	*CSF2RA, IL3RA*	CR2
P 23	6	F	7.7	46,XX	IR	2	—	CR1

Abbreviations: CR1, complete first remission; F, female; HR, high risk; IR; intermediate risk; M, male; RG, risk group at diagnosis; SR, standard risk; WBC, white blood cell. [a]Spectral karyotyping was performed to resolve the complex karyotype.

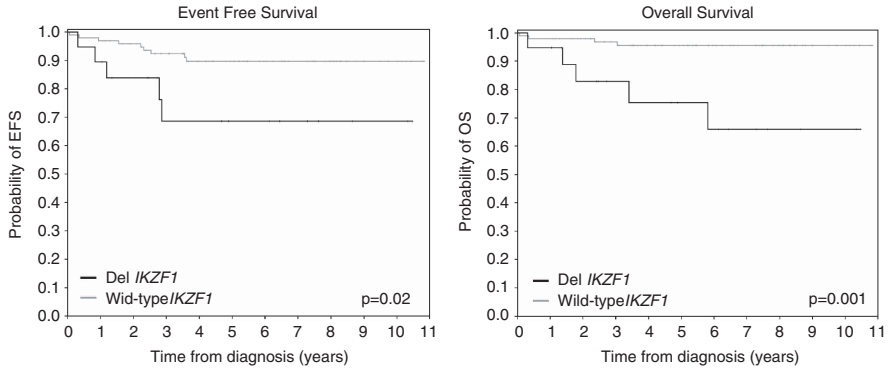

Figure 1. Kaplan-Meier survival plots of 116 BCP ALL cases included in this study and treated according to NOPHO protocols, showing the probabilities of event-free survival (left) and overall survival (right) in cases with deletion of *IKZF1* (black line) and wild-type *IKZF1* (gray line).

isolated cases,[13,14] which indicates that this aberration could escape detection using high-resolution arrays.

In the group with *IKZF1* deletions, both pEFS and pOS were significantly reduced compared with the group with intact *IKZF1* ($P = 0.02$ and $P = 0.001$, respectively) (Figure 1). Five events were recorded in the *IKZF1*-deleted group (four relapses and one death in first remission) and none of the relapsed patients survived. One case had a complex hyperdiploid karyotype and the remaining four cases showed no risk-stratifying aberrations (Table 1). In a multivariate analysis adjusting for WBC count, age, sex and genetic risk group at diagnosis, *IKZF1* deletion was an independent risk factor regarding both event-free survival (EFS; $P = 0.028$) and overall survival (OS; $P = 0.005$). CNS status was excluded from the multivariate analysis as only one patient had evidence of CNS involvement at diagnosis.

We found that the male to female ratio was 2.2 and the median WBC count 20.3 in the group with *IKZF1* deletions, compared with a gender ratio of 1.0 and a median WBC count of 8.1 in the *IKZF1* wild-type group. The risk-group distribution was similar to the one of the general cohort, although the *IKZF1*-deleted group had a slightly larger proportion of high-risk patients compared with the group with intact *IKZF1* (21% and 15%, respectively).

In the group of patients who lacked risk-stratifying aberrations at diagnosis (34/116), *IKZF1* deletions were detected in 26% (9/34) of cases. Four patients died in this group and they all harbored *IKZF1* deletions (P7, P8, P16, P19). The only patient that relapsed and survived carried an amplification of *PAX5* and a deletion of *CSF2RA* and *IL3RA*, but no *IKZF1* deletion.

The frequency of *IKZF1* deletions in our material was consistent with previous reports of unselected BCP ALL cohorts, despite the fact that *BCR-ABL1*-positive ALL was underrepresented in our cohort. We found that cases harboring *IKZF1* deletions had an inferior outcome in terms of EFS and in particular as regards OS. These results are in agreement with previous studies showing a strong correlation between the presence of *IKZF1* deletions and inferior outcome.[3,5] We also observed a high prevalence of *IKZF1* deletions in cases that lacked stratifying aberrations at diagnosis. Notably, *IKZF1* deletions were detected in all cases that did not survive the disease in this group (4/34) and only one of these cases had been assigned to the high-risk group at diagnosis. This highlights the potential additional value of investigating the presence of *IKZF1* deletions in the genetic risk assessment of BCP ALL. Our results indicate that *IKZF1* deletion represents a negative prognostic marker for childhood leukemia treated according to NOPHO protocols. The finding of five patients with intragenic amplifications of *PAX5* warrants further studies to determine the true frequency and potential prognostic impact of this novel aberration in larger cohorts.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Nilotinib treatment-associated accelerated atherosclerosis: when is the risk justified?

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Atherosclerosis is the leading cause of death and morbidity in developed countries and is the culprit behind coronary artery disease (CAD), cerebral vascular disease (CVD) and peripheral artery occlusive disease (PAOD). Atherosclerosis leads to segmental narrowing and occlusion of arteries, and current opinion favors a complex pathogenetic process that involves the endothelium, platelets, monocytes/macrophages, neutrophils, dendritic cells, T and B lymphocytes, lipids, inflammation and chemokines/cytokines.

Two papers in *Leukemia* recently reported the prevalence of PAOD in tyrosine kinase inhibitor (TKI)-treated patients with chronic myeloid leukemia (CML)[1,2] Conflict of interest statements declared 'editorial assistance' from Novartis pharmaceuticals (manufacturer of nilotinib and imatinib) for one of the reports[2] My comments will focus on the other report by Kim *et al.*,[1] who prospectively screened 129 CML patients for pathological PAOD, using ankle-brachial index (ABI). Pathological PAOD (defined by <0.9 ABI) was documented in 6.3% of patients receiving imatinib as first-line therapy, 26% receiving nilotinib as first-line therapy and 35.7% receiving nilotinib as second-line therapy ($P<0.05$). Clinically overt PAOD was seen in five patients, all of whom were exposed to nilotinib therapy. The detrimental effect of nilotinib was evident despite a shorter duration of treatment (median 30 vs 102 months for imatinib). Cardiovascular risk factors were similar between the two groups.

In the second part of their study, Kim *et al.*[1] reviewed 27 cases of TKI treatment-associated overt PAOD accrued from several collaborating centers and discovered that all but one of these patients were exposed to nilotinib therapy, including 20 patients who were receiving nilotinib as first- or second-line treatment of CP-CML. These events were severe enough to require percutaneous transluminal angioplasty in 33.3% of the cases, stent implantation in 22.2%, amputation in 22.2% and surgery in 18.5%.

The observations from Kim *et al.*[1] are consistent with those of earlier[3,4] and more recent[5,6] reports associating nilotinib with accelerated atherosclerosis. Aichberger *et al.*[3] reported a 33% incidence of PAOD, myocardial infarction, spinal infarction or subdural hematoma, among 24 CML patients treated with nilotinib. Tefferi *et al.*[4] described two patients who experienced sudden death or severe PAOD/CAD; continued nilotinib treatment in the latter patient was associated with rapid progression of intra- and extracranial atherosclerosis leading to stroke.[6] Most recently, Levato *et al.*[5] reported their single-institution experience with 82 CML patients treated with imatinib ($n=55$) or nilotinib ($n=27$); four (14.8%) nilotinib-treated patients developed severe PAOD or other vascular disease. In contrast, none of the 55 imatinib-treated patients developed PAOD and only one experienced myocardial infarction, despite a longer median duration of treatment with imatinib (79.5 months) vs nilotinib (21.5 months).

Taken together, the above observations strongly implicate nilotinib therapy as being proatherogenic. Regardless of what the underlying mechanisms for this might be, the question is whether or not it is necessary or appropriate to subject newly diagnosed patients with CP-CML to this risk, considering the remarkable efficacy and safety of imatinib therapy. The 6-year follow-up of 553 imatinib-treated patients in the first international randomized study (the IRIS study) showed an overall complete cytogenetic remission (CCyR) rate of 83% and overall (OS) and progression-free (PFS) survival of 88 and 93%, respectively. PFS was higher ($>95\%$) in patients achieving CCyR or partial (PCyR) cytogenetic remission (corresponding to BCR–ABL1 transcripts of $<10\%$) at 6 months.[7] Disease progression after the first 3 years of treatment was unusual. The majority of the patients assigned to the imatinib arm of the IRIS study have remained on the drug long-term.

The observations from the IRIS study were similar to those of many other studies, including a single-institution study of 204 CP-CML patients receiving imatinib as first-line therapy; 5-year follow-up with full event accounting revealed CCyR of 82.7%, major molecular response (MMR) of 50.1%, OS of 83.2%, PFS of 82.7% and imatinib discontinuation rate of 25%.[8] As was the case in the IRIS study, CCyR was crucial for improved survival but achieving MMR over and above CCyR conferred no further advantage. In yet another large-scale study of imatinib therapy in newly diagnosed CP-CML, survival was similar in CCyR patients with ($<0.01\%$ BCR-ABL1 transcripts) or without (0.1 to $<1\%$ BCR-ABL1 transcripts) MMR.[9]

The importance of close monitoring of response to imatinib therapy and the possibility of early identification of suboptimal responders with inferior long-term outcome has been addressed by multiple studies and highlighted in a recent report of 1303 patients with CP-CML receiving frontline imatinib therapy.[10] In the particular study, BCR–ABL1 transcripts at 3 months decreased to $\leqslant 1\%$ in 31% of the patients, to >1–10% in 41% and remained $>10\%$ in 28%; the corresponding 5-year OS were 97, 94 and 87% ($P<0.05$).[10] Similarly, 5-year OS was 95% in patients with at least PCyR (73% of the patients) vs 87% otherwise.[10] At 6 months, BCR–ABL1 transcripts remained $>1\%$ (that is, no CCyR) in 37% of the patients, and 5-year OS was 89% in this group of patients vs 97% for the 63% of patients achieving $\leqslant 1\%$ transcript level (that is, CCyR).[10]

For patients who do not tolerate imatinib or show resistance to it, several second generation TKIs (SG-TKI) have been developed and some have recently been approved for clinical use (nilotinib, dasatinib, bosutinib and ponatinib). These drugs are usually more potent than imatinib and are able to effectively substitute for it in case of drug intolerance and also offer an alternative to allogeneic stem cell transplant in case of drug resistance. The question is

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