

Copy number alterations define outcome in Philadelphia chromosome-positive acute lymphoblastic leukemia

The introduction of tyrosine kinase inhibitor (TKI) imatinib has improved outcome of Philadelphia chromosome-positive (Ph+) acute lymphoblastic leukemia (ALL), and the second- and third-generation TKI, such as dasatinib or ponatinib, may prove even more effective.¹ Unfortunately, treatment failures remain frequent, often due to the emergence of *BCR-ABL1* kinase domain (KD) mutations. The second-generation TKI were developed to overcome most imatinib-resistant KD mutations. However, several mutations, such as T315I and F317I/L, evade also the second-generation TKI, and highly-resistant compound mutations even the third.² In addition, *IKZF1* deletions, especially in combination with deletions in *PAX5* and/or *CDKN2A/B* genes, may define a group with unfavorable outcome (“IKZF1 plus”).³ Considering that the first trials of chemotherapy-free treatment regimens in Ph+ ALL have shown promising results,⁴ and that TKI-based therapies induce durable remissions in some patients even without transplantation,⁵ identification of prognostic and predictive markers is of utmost importance for treatment stratification.

Here, we investigated potential biomarkers for treatment outcome in a retrospective, nationwide Ph+ ALL adult population. In addition to assessing clinical parameters, we sequenced diagnostic and relapse-phase patient samples with a targeted next-generation sequencing (NGS) gene panel consisting of 75 leukemia-associated genes. We also analyzed copy-number alterations (CNA) in *IKZF1*, *PAX5*, and *CDKN2A/B* genes. As T315I kinase domain mutations cause broad resistance to TKI,² we examined the prevalence of subclonal T315I with digital droplet polymerase chain reaction (ddPCR). All clinical data was obtained from the Finnish Hematology Registry (FHR), a population-based centralized database, which stores data on clinical variables, treatments, treatment outcomes, laboratory values, and results from cytogenetic and molecular analyses. Bone marrow samples were retrieved from the clinical laboratories and from the Finnish Hematology Registry and Clinical Biobank (FHRB; <https://www.fhrb.fi/>). All patients signed a written informed consent. The study was approved by the Helsinki University Hospital Ethical Committee, and it was conducted in accordance with the Declaration of Helsinki. FHR contained data of 141 Ph+ ALL adult patients (years 1984-2020). A total of 82 patients had received TKI-based therapies first-line and were selected for the biomarker analyses. The median overall survival (OS) of was 87.6 months (95% confidence interval [CI]: 51.3-169.8; n=82). Of the 82 TKI-era patients, 36 received CVAD, nine

CVAD+pegasparaginase, 18 MEA (mitoxantrone+etoposide+cytarabine) and 13 NOPHO ALL-2008 non-HR as an induction regimen. Six patients received other/customized induction treatments, and of them three were treated solely with steroids and TKI. Two of the patients who were treated with TKI and steroids succumbed to leukemia rapidly, but one patient is still alive, more than 6 years from the diagnosis. The outcome of imatinib (n=43) and dasatinib-treated (n=39) patients did not differ statistically. For imatinib-treated patients, 3-year and 5-year OS estimates were 67% and 58%, respectively, and for dasatinib-treated patients 64% and 51%. Allogeneic hematopoietic stem cell transplantation (alloHSCT) was associated with better outcome in the imatinib (*Online Supplementary Figure S1A and B*), but not in the dasatinib-treated patients (*Online Supplementary Figure S1C and D*), even though the dasatinib-treated, non-allotransplanted patients were significantly older than the transplanted patients (median age 58; range, 28-79 years vs. 41 years; range, 20-69 years; $P=0.009$, Wilcoxon signed-rank test). Presently, Finnish Leukemia Group recommends dasatinib as frontline TKI in adult Ph+ ALL. Therefore, the dasatinib-treated patients in this study reflect a more modern treatment era (median year of diagnosis 2015; range, 2009-2020 vs. 2006; range, 2001-2020; $P<0.0001$, Wilcoxon signed-rank test), which may partly explain the difference in survival. In a single prospective randomized study, dasatinib had significant survival benefit compared to imatinib.¹ In addition, the benefit of alloHSCT was no longer evident in a dasatinib-treated population.^{3,5} Factors that favor dasatinib over imatinib include more potent and broader kinase inhibition, blood-brain barrier penetration, and possible anti-leukemic immunomodulatory effects.⁶ In total, 43 of the TKI-treated patients (52%) were allotransplanted. The non-allotransplanted patients were expectedly older (median age 64 years; range, 28-80 years vs. 42 years; range, 19-69 years; $P<0.0001$, Wilcoxon signed-rank test), but the groups did not otherwise differ significantly. AlloHSCT was associated with better outcome (*Online Supplementary Figure S2A and B*), but after excluding elderly (age >65 years) patients, the survival advantage disappeared (n=63; *Online Supplementary Figure S2C and D*). This exclusion was done to allow more realistic comparison, as over 65-year-olds are in most cases considered ineligible for alloHSCT. Especially in a retrospective, real-life cohort, allotransplanted and non-transplanted patients represent differing entities, with the tendency of the non-transplanted patients to be older and more often non-eligible for intensive treatment modal-

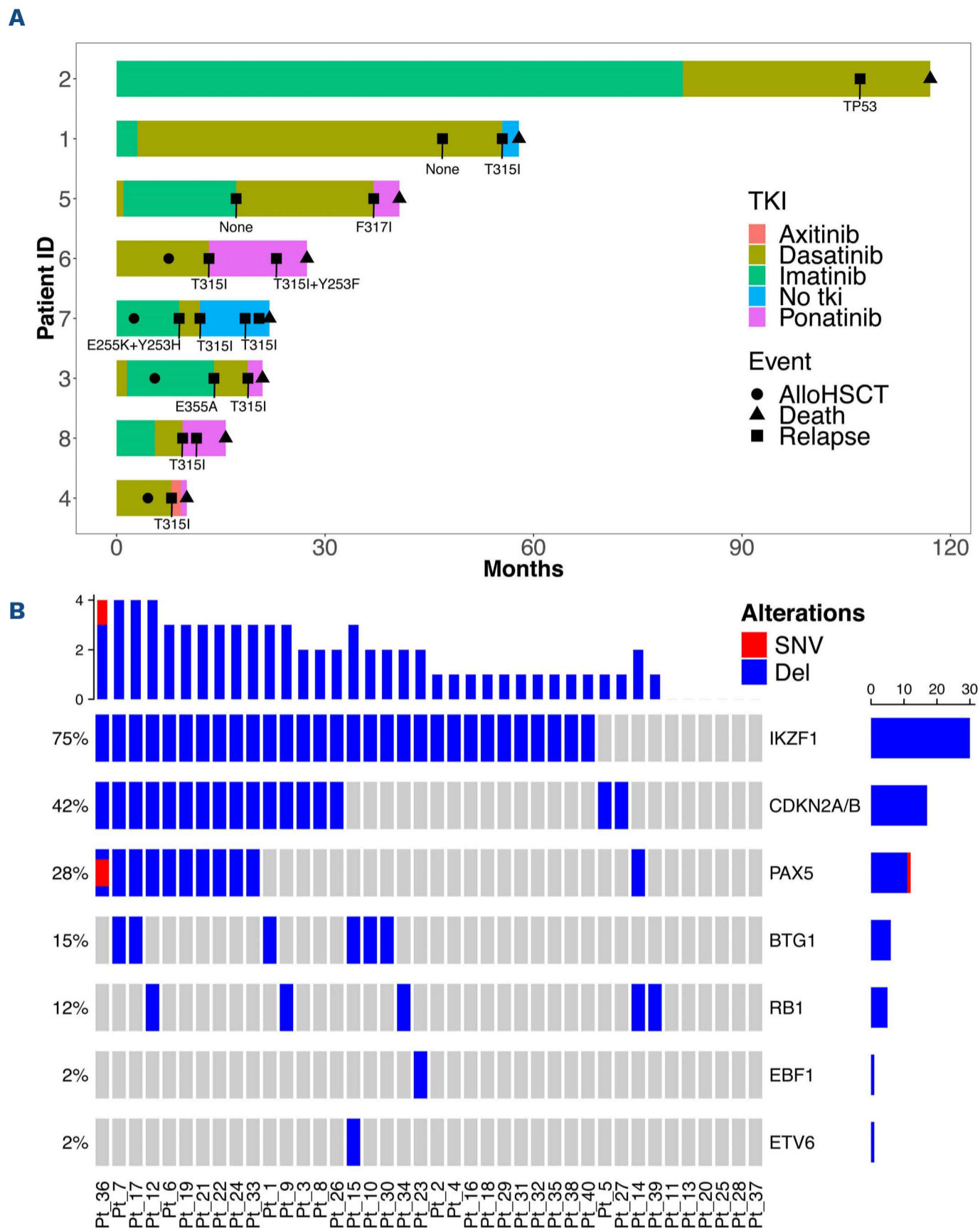


Figure 1. Detected mutations in the analyzed samples. (A) The detected mutations in the relapse-phase samples and their relation to the given tyrosine kinase inhibitor treatment. Timeline starting from the diagnosis. For the T315I digital droplet polymerase chain reaction (ddPCR) assay, RNA was extracted using a QIAamp RNA Blood Mini kit (Qiagen, Hilden, Germany) and 2 μ g was converted to cDNA using a SuperScript VILO cDNA Synthesis Kit (ThermoFisher, Waltham, MA) according to the manufacturer's protocol. A 40 cycle PCR amplification was performed with a forward primer located in BCR exon 1 and reverse primer in ABL1 exon 10,² using Q5 High Fidelity DNA polymerase (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. ddPCR was performed on the 4 dilutions (10^5 to 10^8) using ddPCR Supermix for Probes on a QX200 ddPCR system (Bio-Rad, Hercules, CA) with forward primer: GGTCTGCACCCGGGAG, reverse primer: AGGTAGTCCAGGAGGTTTC, wild-type probe: HEX-CCGTTCTA-TATCATCACTGAGTTCATGACCTAGAACG-BHQ1 and T315I probe: FAM-CCGTTCTATATCATCA_tTGAGTTCATGACCTAGAACGG-BHQ1. Cycling conditions were 95°C for 10 minutes, followed by 40 cycles of 94°C for 30 seconds and 60°C for 60 seconds. (B) The detected mutations in the diagnosis-phase samples. Copy-number alterations in *IKZF1*, *CDKN2A/B*, *PAX5*, *EBF1*, *ETV6*, *BTG1*, and *RB1* genes were detected with SALSA MLPA Probemix P335 ALL-IKZF1 kit (MRC Holland, Amsterdam, the Netherlands). The assay was performed according to the manufacturer's protocol and the data were analyzed with Coffalyser.Net software (MRC Holland, Amsterdam, the Netherlands). Both diagnosis and relapse-phase samples were analyzed with a targeted next-generation sequencing gene panel consisting of 75 leukemia-associated genes. 150 ng of genomic DNA was processed according to SeqCap EZ HyperCap Workflow User's Guide, v2.1 Dec 2017 Enzymatic Fragmentation (Kapa Biosystems, Inc., Wilmington, MA, USA) using

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Unique Dual Index adapters by IDT (Integrated DNA Technologies, Coralville, IA, USA). Library quality check was performed using LabChip GX Touch HT High Sensitivity assay (PerkinElmer, USA). 7 cycles were used for precapture amplification. SeqCap custom captures (170621_HG38_ALL-75G_EZ_HX3) were performed in 6-7 samples multiplexed DNA Sample Library Pools using 600 µg of each library. 10 cycles were used for post capture amplification. The captured library pools were quantified for sequencing using KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA, USA) and 2100 Bioanalyzer High sensitivity kit. The samples were sequenced in 3 batches. The first batch was sequenced with Illumina HiSeq2500 system in HiSeq high output mode using v4 kits (Illumina, San Diego, CA, USA). Read length for the paired-end run was 2x101 bp. The following batches were sequenced with Illumina NovaSeq system using S4 flow cell with lane divider (Illumina, San Diego, CA, USA) and v1.0 chemistry. Read length for the paired-end run was 2x101.

ities, making direct comparison difficult. After the exclusion, the non-allotransplanted patients were still significantly older than the transplanted ones (median 54 years; range, 28-64 years vs. 42 years; range, 19-64 years; $P=0.003$, Wilcoxon signed-rank test). The patient characteristics between these two cohorts did not otherwise differ significantly. In the non-allotransplanted patients, 27% (7/26) of deaths

were caused by non-leukemia-related reasons (such as heart failure, gastric cancer, breast cancer), 50% (13/26) were due to relapse or primary refractory disease, and 23% (6/26) were treatment-related. In the allotransplanted cohort, 65% (11/17) of the deaths were due to transplantation-related causes and 29% (5/17) due to a relapse. One death was due to other causes. In the competing risks analysis, the proportion of both leukemia-re-

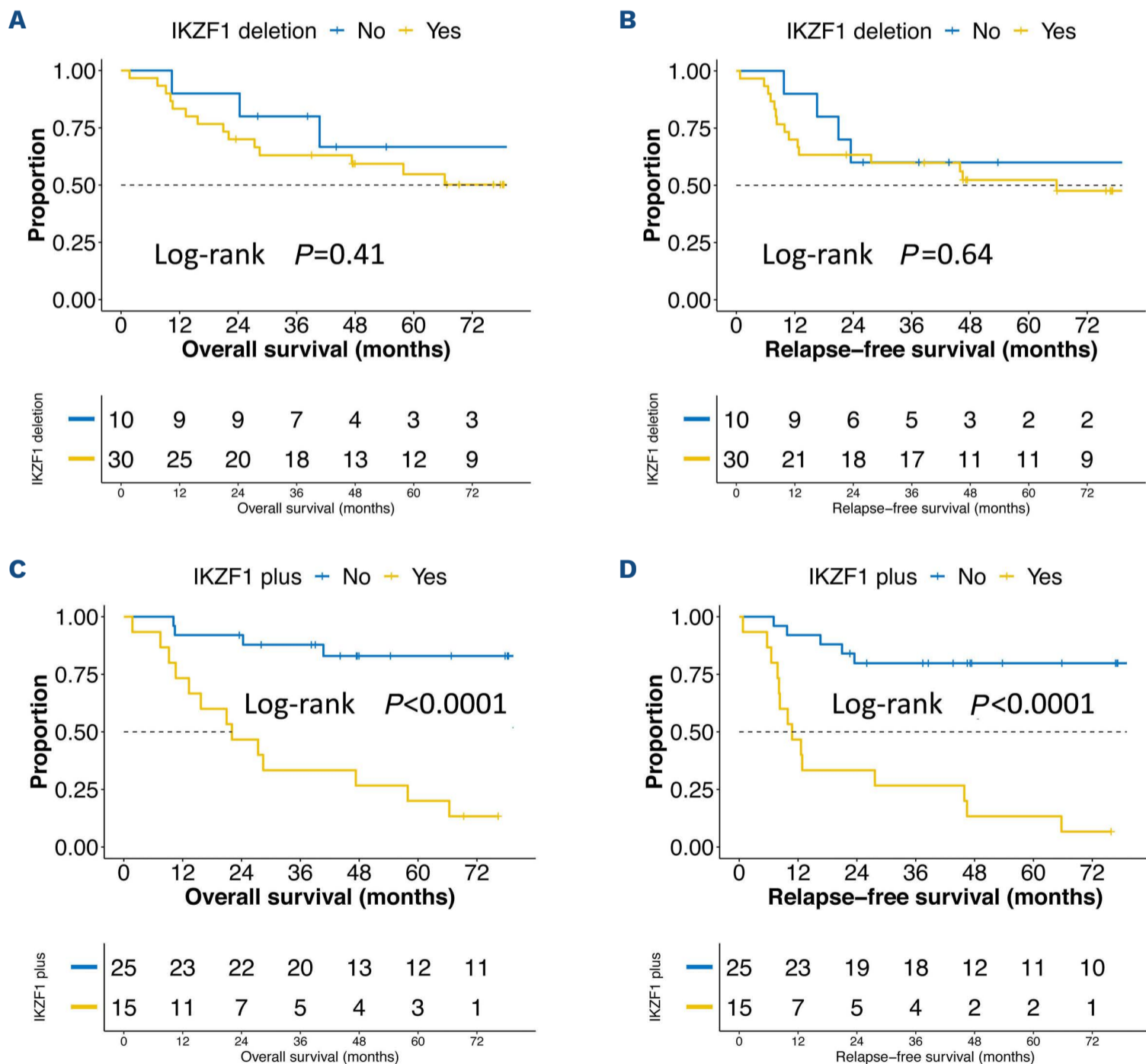


Figure 2. IKZF1 plus genotype predicts poor survival. (A) Overall survival and (B) relapse-free survival of patients according to the presence of *IKZF1* deletion. (C) Overall survival and (D) relapse-free survival of patients according to the presence of *IKZF1* plus (*IKZF1* deletion with *CDKN2A/B* and/or *PAX5* deletion). Events after 80 months are not shown. Kaplan-Meier estimate, log rank test.

Table 1. Univariate Cox regression analysis of overall and relapse-free survival in first-line tyrosine kinase inhibitor-treated patients.

	Univariate analysis for OS HR (95% CI)	P	Univariate analysis for RFS HR (95% CI)	P	N of observations
Age, continuous	1.04 (1.02-1.06)	0.0004	1.04 (1.01-1.06)	0.001	82
Age, categorical (>65 y vs. ≤ 65 y)	3.32 (1.75-6.26)	<0.0001	3.04 (1.63-5.67)	0.0002	82
AlloHSCT	0.45 (0.24-0.82)	0.008	0.51 (0.28-0.93)	0.03	82
Dasatinib vs. imatinib	1.16 (0.60-2.24)	0.7	1.21 (0.64-2.28)	0.6	82
WBC	1.003 (0.999-1.007)	0.2	1.003 (0.998-1.007)	0.2	79
WBC ≥30x10 ⁹ /L	1.81 (0.98-3.37)	0.06	1.76 (0.96-3.23)	0.06	79
WBC ≥50x10 ⁹ /L	1.77 (0.89-3.50)	0.1	1.64 (0.83-3.23)	0.1	79
Hemoglobin	1.002 (0.990-1.014)	0.7	1.003 (0.99-1.02)	0.6	78
Platelets	0.998 (0.993-1.002)	0.3	0.998 (0.993-1.002)	0.3	78
LDH	1.00 (0.9997-1.001)	0.5	1.00 (0.9998-1.001)	0.5	74
BM blast%	1.02 (0.99-1.06)	0.2	1.03 (0.99-1.06)	0.1	53
P210 vs. P190, transcript type	0.50 (0.17-1.46)	0.2	0.61 (0.23-1.64)	0.3	53
ACA (karyotype)	1.05 (0.48-2.27)	0.9	0.92 (0.43-1.93)	0.8	54
MRD-negative in 3 months, no vs. yes	0.65 (0.32-1.33)	0.2	0.61 (0.31-1.21)	0.2	68
<i>IKZF1</i> deletion	1.59 (0.52-4.84)	0.4	1.27 (0.46-3.46)	0.6	40
<i>IKZF1</i> plus	8.37 (2.71-25.83)	<0.0001	8.56 (3.04-24.07)	<0.0001	40

*OS: overall survival; RFS: relapse-free survival; HR: hazard ratio; CI: confidence interval; y: years; AlloHSCT: allogeneic hematopoietic stem cell transplantation; WBC: white blood cell count; LDH: lactate dehydrogenase; BM: bone marrow; ACA: additional chromosomal abnormalities; MRD: minimal residual disease.

lated deaths ($P=0.02$, Gray's test) and other than leukemia or treatment-related causes of death ($P=0.02$, Gray's test) were more common in the non-allotransplanted patients. A proportion of Ph+ ALL patients experience prolonged survival with TKI-based therapies only,⁷ and many die due to non-leukemia-related causes. TKI modulate the immune system, and part of their effect might stem from overcoming the immunosuppressive state in the leukemic bone marrow.⁶

The targeted NGS panel detected only a single missense *PAX5* mutation (p.V26G) in the diagnostic samples ($n=41$) and no point mutations in the *IKZF1* or *CDKN2A/B* genes. In the relapse-phase samples ($n=11$), *ABL1* mutations causing TKI resistance were frequent (p.T315I, $n=5$; p.E355A, $n=1$; p.F317I, $n=1$; p.T315I+p.Y253F, $n=1$; p.E255K+p.Y253F, $n=1$; Figure 1A). In addition, one *TP53* mutation (p.C176F) was detected in a relapse-phase sample. The variant calling was performed as previously described.⁸ As especially T315I mutations possess clinical significance, we designed a ddPCR assay capable of detecting T315I down to 0.04% variant allele frequency (VAF). Altogether 32 samples (26

pretreatment, 6 relapse-phase) from 25 patients were examined. Contrary to previous studies, where up to 25% of Ph+ ALL patients have been reported to harbor pretreatment T315I subclones,⁷ only one baseline sample tested positive for T315I with a VAF of 0.10%. The patient was treated with imatinib but relapsed 9 months later with an E255K mutation.² Imatinib was switched to dasatinib, followed by a relapse with T315I 2 months later. In line with our results, Short *et al.* reported only a single pretreatment T315I mutation in 63 Ph+ ALL samples using highly accurate duplex-sequencing.⁹ Importantly, these baseline mutations did not correlate with treatment success. In view of these data, screening for pretreatment T315I seems unwarranted. Our ddPCR assay detected T315I mutations in five of six of the relapse phase samples, all with a VAF >25%. All these mutations were also detected by clinical routine mutation analysis and the NGS panel. The detected mutations from the relapse phase samples are collected to Figure 1A.

Most Ph+ ALL patients harbor *IKZF1* deletions, although their role in the risk stratification remains undecided.^{3,10}

Using multiplex ligation-dependent probe amplification analysis we detected *IKZF1* deletions in 75% (n=30), *CDKN2A/B* deletions in 42% (n=17), and *PAX5* deletions in 28% (n=11) of the analyzed samples (n=40). Other candidate gene alterations detected by the assay were less frequent (*EBF1*, n=1; *ETV6*, n=1; *BTG1*, n=6; *RB1*, n=5). In 38% of cases, *IKZF1* deletion was accompanied by a deletion in *CDKN2A/B* and/or *PAX5* genes (*IKZF1* plus). The detected mutations from the diagnosis phase samples are shown in Figure 1B. The presence of *IKZF1* deletions alone had no effect on survival (Figure 2A and B), whereas *IKZF1* plus genotype lead to inferior prognosis (Figure 2C and D). Within the *IKZF1* plus group, alloHSCT did not improve survival, although the cohort size is limited (*Online Supplementary Figure S3A and B*). Cumulative incidence for relapse was 56% at 12 months after alloHSCT in *IKZF1* plus patients and only 7% in non-plus patients (*Online Supplementary Figure S3C*). Non-relapse mortality did not differ between these two cohorts (*Online Supplementary Figure S3D*). *IKZF1* plus patients had higher white blood cell count at diagnosis, but the cohorts did not otherwise differ significantly at baseline. When analyzing all first-line TKI-treated patients, in Cox regression univariate analyses *IKZF1* plus genotype, age, and alloHSCT were the only significant predictors for both OS and relapse-free survival (Table 1).

No consensus currently exists how treatment of *IKZF1* plus patients should be modified. AlloHSCT may not improve survival in this group.³ Limited data indicate that a combination of dasatinib and blinatumomab might prove beneficial.⁴ In the younger patients, a modern MRD-driven intensive chemotherapy protocol seemed effective.¹⁰ *IKZF1* plus patients may be primary resistant at the progenitor/stem cell level to TKI-based therapies and more detailed mechanistic studies may give insight to effective treatment alternatives.¹¹ Retinoids, immunomodulatory drugs, crizotinib, and a combination of asciminib and ponatinib are currently being investigated and may provide an alternative also for the elderly patients and those non-eligible for intensive therapies.¹²⁻¹⁵ To conclude, testing for CNA should be implemented in the routine diagnostics of Ph+ ALL. *IKZF1* plus genotype constitutes a high-risk group, which may benefit from immuno-oncological or intensified treatment approaches.

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Disclosures

TS (not related to this study) is a member of the advisory board of Celgene and AbbVie; is a member of the advisory board of and received lecture fees from Pfizer and Janssen-Cilag; received lecture fees from Bristol Myers Squibb; received congress fees from and is a member of the advisory board of Novartis; received congress fees from Amgen. MP (not-related to this study) is a member of the advisory board of Pfizer and AbbVie; received lecture and congress fees from Novartis. OB received consultancy fees from Novartis and Sanofi. SM (not related to this study) received research funding from Novartis, BMS, Janpix, and Pfizer. All other authors have no conflicts of interest to disclose.

Contributions

HH, NP, PE, SM and KP developed the concept and design of the study; HH, TS, MSä, MSi, MP, MI-R, PK, EE and KP collected and assembled data; HH, NP, MK, OB and KP analyzed and interpreted the data. All authors wrote the manuscript and gave their approval of the final version.

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Data-sharing statement

The datasets generated and analyzed for the current study can be shared upon request to the corresponding author. All data will be shared in an anonymized form, as regulated by the General Data Protection Regulation (GDPR) of the European Union.