Genetic and genomic analysis of acute lymphoblastic leukemia in older adults reveals a distinct profile of abnormalities: analysis of 210 patients from the UKALL14 and UKALL60+ clinical trials

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Abstract

Despite being predominantly a childhood disease, the incidence of acute lymphoblastic leukemia (ALL) has a second peak in adults aged 60 years and over. These older adults fare extremely poorly with existing treatment strategies and very few studies have undertaken a comprehensive genetic and genomic characterization to improve prognosis in this age group. We performed cytogenetic, single nucleotide polymorphism (SNP) array and next-generation sequencing (NGS) analyses on samples from 210 patients aged ≥60 years from the UKALL14 and UKALL60+ clinical trials. *BCR-ABL1*-positive disease was present in 26% (55/210) of patients, followed by low hypodiploidy/near triploidy in 13% (28/210). Cytogenetically cryptic rearrangements in *CRLF2*, *ZNF384* and *MEF2D* were detected in 5%, 1% and <1% of patients, respectively. Copy number abnormalities were common and deletions in ALL driver genes were seen in 77% of cases. *IKZF1* deletion was present in 51% (40/78) of samples tested and the *IKZF1*^{plus} profile was identified in over a third (28/77) of cases of B-cell precursor ALL. The genetic good-risk abnormalities high hyperdiploidy (n=2), *ETV6-RUNX1* (no cases) and *ERG* deletion (no cases) were exceptionally rare in this cohort. RAS pathway mutations were seen in 17% (4/23) of screened samples. *KDM6A* abnormalities, including biallelic deletions, were discovered in 5% (4/78) of SNP arrays and 9% (2/23) of NGS samples, and represent novel, potentially therapeutically actionable lesions using EZH2 inhibitors. Outcome remained poor with 5-year event-free and overall survival rates of 17% and 24%, respectively, across the cohort, indicating a need for novel therapeutic strategies.

Introduction

Acute lymphoblastic leukemia (ALL) presents most commonly in early childhood.[1] However, the disease has a bimodal incidence with a second smaller peak in adults aged 60 years old and over.[2] Optimal care of these older adults (≥60 years) remains an area of unmet clinical need. Although they account for only 30-35% of diagnoses each year in adults, around 60% of disease-related deaths occur within this age group, and they are the only ALL patients not to have benefitted from the stepwise improvements in prognosis driven through successive clinical trials in children and younger adults.[2,3]

Primary chromosomal abnormalities are one of the hallmarks of ALL and greatly influence treatment decisions and prognosis.[4,5] Although *BCR-ABL1* is well recognized as the most common genetic subgroup in adult ALL,[5] to date, only limited biological characterization of older patients beyond the conventionally defined risk groups has been performed. One recent study provided a comprehensive genomic profile of 1,988 subjects with B-cell precursor (BCP)-ALL using a combination of transcrip-

tome, whole genome and exome sequencing and identified 23 genetic subtypes.[6] Despite this impressive cohort, only 103 patients aged 60 years and over at diagnosis were included. Such efforts focused on older individuals are needed to improve prognostication and to identify novel therapeutic targets.[7,8]

A proportion of patients do not harbor a cytogenetically visible disease-defining lesion, but have a gene expression profile similar to that of *BCR-ABL1*-positive disease (Ph-like/*BCR-ABL*-like ALL).[9,10] Approximately 50% of children and young adults with this entity have cytogenetically-cryptic *IGH-CRLF2* or *P2RY8-CRLF2* rearrangements, which activate JAK-STAT signaling.[11,12] Other recurrent gene rearrangements include ABL-class fusions (affecting *ABL1*, *ABL2*, *PDGFRB* or *CSF1R*) in 9-13% of Ph-like cases and the JAK-STAT pathway activating rearrangements of *JAK2* or *EPOR* in 7-10% and 3-6% of patients, respectively.[11,12] To date, most studies have focused on pediatric and young adult (<60 years) cohorts,[11,13] although one study found the Ph-like signature in 24% of older BCP-ALL patients in a restricted subgroup lacking large scale aneuploidy.[12] Separately, *ZNF384* and *MEF2D* rearrangements have been reported in 2-6% of pediatric BCP-ALL cases, and form distinct clinical entities.[14,15]

Focal copy number abnormalities (CNA) frequently target genes that are involved in B-cell development or cell cycle regulation These secondary abnormalities drive transformation of a pre-leukemic clone into overt disease and include deletions of *EBF1* on 5q33.3, *IKZF1* on 7p12.2, *CDKN2A* and *CDKN2B* on 9p21.3, *PAX5* on 9p13.2, *ETV6* on 12p13.2, *BTG1* on 12q21.33 and *RB1* on 13q14.2.[16] Importantly, particular combinations of CNA have an impact on prognosis.[17-19] The *IKZF1*^{plus} profile is based on the co-occurrence of *IKZF1* deletion with deletions of *CDKN2A*, *CDKN2B*, *PAX5* or the pseudoautosomal region 1 (PAR1) on Xp22.33/Yp11.31 (resulting in *P2RY8-CRLF2* fusion) in the absence of *ERG* deletion.[17] This copy number profile is associated with a significantly poorer outcome in childhood ALL patients, highlighting the prognostic importance of large-scale copy number analyses.

To date, profiling the genetic and genomic landscape of ALL has been primarily restricted to younger patients with few analyses focused on older individuals. Here, we applied cytogenetic, copy number and next-generation sequencing (NGS) techniques to investigate whether the primary and secondary genetic abnormalities of ALL in older adults are distinct from those encountered in their younger counterparts. We additionally sought to identify novel druggable targets, a particular priority for such patients because of the high toxicity and low success rates of traditional chemotherapeutic approaches in this group.[20]

Methods

Patients and samples

Patients aged 60 years and over were identified for genetic profiling studies from two large UK-wide multicenter clinical trials (UKALL14 and UKALL60+). The UKALL14 study is registered as NCT01085617 (*https://www.clinicaltrials.gov/ct2/show/NCT01085617*), and the UKALL60+ study is registered as NCT01616238 *https://clinicaltrials.gov/ct2/show/NCT01616238*) (*Online Supplementary Methods*).

Baseline cytogenetic analyses, typically consisting of a diagnostic karyotype and fluorescence *in situ* hybridization (FISH) for *BCR-ABL1* fusion and *KMT2A* translocations, were performed in accredited diagnostic genetic laboratories throughout the UK and then centrally reviewed and entered into the Leukaemia Research Cytogenetics Group database.

The study was approved by the institutional review board of each treatment center and all patients gave written informed consent for data collection and genetic studies as specified by the trials' protocols.

Detection of primary genetic subgroups

Diagnostic karyotype and FISH results from the regional genetic centers were first examined and patients were coded into one of six subgroups: *BCR-ABL1*, *TCF3-PBX1*, *KMT2A* fusions, high hyperdiploidy (51-65 chromosomes), low hypodiploidy/near triploidy (HoTr) (30-39 or 60-78 chromosomes) or T-cell ALL (T-ALL). Reverse transcriptase polymerase chain reaction analysis was performed to identify the presence of Bcr-Abl p190, p210 and p230 oncoproteins in *BCR-ABL1*-positive cases. Next, all BCP-ALL cases lacking a primary chromosomal abnormality, hereafter termed B-other ALL, were identified. B-other cases with available fixed cell samples were further investigated by the Leukaemia Research Cytogenetics Group to determine the occurrence of cytogenetically cryptic abnormalities using dual color break-apart FISH probes for *CRLF2*, *PDGFRB/CSF1R*, *ABL2*, *IGH*, *ZNF384*, *MEF2D* (Cytocell, Cambridge, UK) and *JAK2* (Kreatech Diagnostics, Amsterdam, the Netherlands). Separately, multiplex ligation-dependent probe amplification (MLPA) using the IKZF1-P335 kit (MRC Holland, the Netherlands) was performed on cases with available DNA as previously described[21] and permitted the detection of the *P2RY8-CRLF2* fusion that occurs through PAR1 deletion on Xp22.33/Yp11.31.

Copy number analysis

Single nucleotide polymorphism (SNP) arrays were performed on DNA extracted from diagnostic bone marrow samples obtained at trial enrolment. SNP arrays were performed at the Newcastle Genomics Centre, Newcastle-upon-Tyne Hospitals NHS Foundation Trust using the

Affymetrix Cytoscan HD (Affymetrix, Santa Clara, CA, USA) or Illumina CytoSNP 850k (Illumina, San Diego, CA, USA) arrays according to the manufacturers' protocols.
Deletions in *IKZF1*, *CDKN2A*, *CDKN2B*, *PAX5*, *RB1*, *ETV6*, *EBF1* and *BTG1* ('driver genes') were specifically identified from the SNP array results, with loss of any part of the gene considered significant. All arm-level and focal CNA were then examined to detect recurrent abnormalities.

Next-generation sequencing

Separately, targeted NGS analyses were performed on selected samples using a custom SureSelect XT2 target enrichment kit (Agilent, Santa Clara, CA, USA). Samples were selected based on availability of DNA and the presence of CNA by SNP array. The capture library was designed to target either the coding regions or full sequence of 44 genes that are well known to be implicated in ALL (*Online Supplementary Table S1*). Libraries were prepared in accordance with the manufacturer's protocol and sequenced on the NextSeq 550 (Illumina, San Diego, CA, USA) using 100 bp paired-end chemistry (*Online Supplementary Methods*).

Survival analysis

Survival analysis was restricted to patients enrolled in UKALL14 because all these patients received similar intensive treatment with curative intent.[22] Patients were grouped according to primary chromosomal abnormalities as described previously.[23] Briefly, patients with complex karyotypes, HoTr or JAK-STAT activating rearrangements were classed as very high risk; patients with any *KMT2A* fusions were classed as high risk; patients with *BCR-ABL1* and other kinase-activating fusions were classed as having tyrosine kinase-activating (TKA) abnormalities; all other BCP-ALL patients were classed as standard risk; and T-ALL patients were analyzed separately. All *P*-values were two-sided and, because of multiple testing, values <0.01 were considered statistically significant. All analyses were performed using Intercooled Stata (StataCorp, College Station, TX, USA) and R version 3.4.3 (*http://www.R-project.org*).

Results

Patients' demographics and baseline cytogenetics

We identified a total of 210 patients aged ≥60 years from the UKALL14 (n=95) and UKALL60+ (n=115) clinical trials. The median age of the patients was 64 years (range, 60-83) and 24% (n=50) were over 70 years at diagnosis. The male:female ratio was 1:1. In total, 90% (n=189) had confirmed BCP-ALL and 5% (n=11) had T-cell disease. The remaining 5% (n=10) did not have a diagnostic immunophenotype centrally recorded. Numbers of patients decreased with advancing age but no significant difference was seen in the genetic subgroups represented in different age groups (*P*=0.47) (Figure 1A, B). The most prevalent abnormality was *BCR-ABL1*, present in 28% (55/200) of evaluable patients. Of these, the p210, p190 and p230 isoforms were present in 40% (22/55), 33% (18/55) and <1% (1/55) of patients, respectively. Two patients had both p190 and p210 isoforms identified and the Bcr-Abl isoform was unknown in the remaining 22% (12/55) patients. Low hypodiploidy/near triploidy (HoTr) was the second most prevalent primary chromosomal abnormality and was identified in 14% (28/200) of patients (*Online Supplementary Table S2*) and *KMT2A-v* rearrangements were discovered in a further 6% (12/200) of patients. Of the patients with BCP-ALL, 47% (88/189) did not have a primary chromosomal abnormality identified by routine cytogenetic and FISH analyses performed in regional cytogenetic centers (B-other ALL).
Among the 11 patients with T-ALL, *TLX1* (n=1) and *TLX3* (n=1) rearrangements were identified. The other nine patients either had no rearrangements identified (n=5) or were not tested (n=4).
Individual patients' demographic, clinical and genetic data are shown in *Online Supplementary Table S3*.

Gene rearrangements in patients with B-other acute lymphoblastic leukemia

Patients with B-other ALL included those with normal (n=21), failed (n=25) or complex karyotypes (n=5). Patients with dic(9;12) (n=2), *IGH* translocation (n=5) or other non-subgroup-defining chromosomal abnormalities (n=30) were also included in the B-other category. Fixed cell samples were available for 74% (65/88) of B-other patients and gene rearrangements were identified in 21% (19/65) (Table 2). Not all samples could be screened for all abnormalities due to lack of availability of material for multiple FISH experiments.
CRLF2 rearrangements were identified in 17% (8/48) of successfully screened cases. The *CRLF2* rearrangement partners were *IGH* (n=5), *P2RY8* (n=2) and unknown (n=1). Two additional patients had *P2RY8-CRLF2* fusion identified by MLPA, through the presence of PAR1 deletion (Table 1).
IGH translocations were present in 26% (14/53) of B-other samples tested. Of these, five cases accounted for patients with *IGH-CRLF2* translocations detailed above, and three cases had separate primary genetic abnormalities identified (one *ZNF384* translocation and two *P2RY8-CRLF2* fusions). In the remaining six samples, the *IGH* partners were *CEBPA* (n=1), *CEBPD* (n=1), *CEBPE* (n=1), *BCL2* (n=1) and unknown (n=2). *ZNF384* and *MEF2D* rearrangements were each identified in 8% (3/40) and 3% (1/39) of screened B-other cases, respectively.
In total, *CRLF2*, *IGH*, and *ZNF384* rearrangements were present in 5%, 3% and 1% of the complete patient cohort, respectively (Figure 1C). No variant *ABL1* (0/83), *PDGFRB*

(0/56), *JAK2* (0/53) or *ABL2* (0/52) rearrangements were detected.

Copy number alterations

SNP arrays were performed on diagnostic bone marrow samples from 78 of the 210 patients (49 from UKALL14 and 29 from UKALL60+) using the Illumina CytoSNP 850k (n=51) and Affymetrix Cytoscan HD (n=27) arrays. The SNP array cohort was reasonably representative of the whole cohort of patients, although *BCR-ABL1*-positive patients were slightly over-represented (*Online Supplementary Table S4*).

Deletions were more frequent than gains in all cytogenetic subgroups apart from high hyperdiploidy. Following the exclusion of probable constitutional copy number variations, as described in the *Online Supplementary Methods*, a median of seven deletions (range, 0-52) and one gain (range, 0-29) were seen per patient sample.

In the 68/78 patients without a primary ploidy shift (defined as HoTr and high hyperdiploidy), large deletions on 9p were the most prevalent arm-level CNA, seen in 22% (15/68) of cases (Figure 2, *Online Supplementary Table S5*). An additional copy of the Philadelphia chromosome was present in 12% (8/68) of patients (26% of *BCR-ABL1*-positive cases) and 1q gains and monosomy 7 were each present in 10% (7/68) of samples.

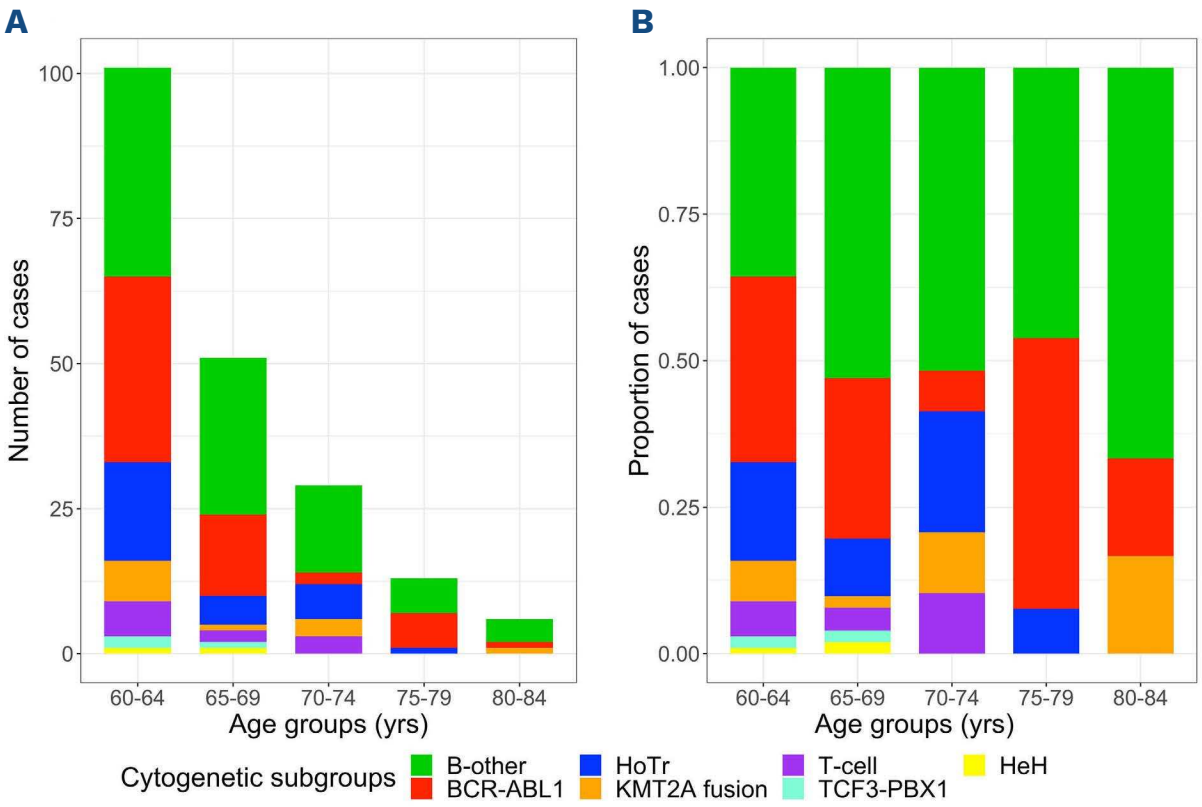
Of the CNA in known driver genes, *IKZF1* deletions were the most frequent abnormality, present in 51% (40/78) of cases. These were focal intragenic deletions in 19 cases, most commonly involving exons 4-7 (n=11) or exons 2-7 (n=4). Rarer *IKZF1* deletions involved exons 4-8 (n=2), exons 2-8 (n=1) and one patient had biallelic *IKZF1* loss involving exons 2-7 and 2-8. Focal *IKZF1* deletions were almost exclusively seen in patients with *BCR-ABL1* (n=13) or *CRLF2* rearrangements (n=5) (*Online Supplementary Table S6*). In the remaining cases, *IKZF1* loss resulted from monosomy 7 (n=16) or del(7p) (n=5) (Figure 2, *Online Supplementary Table S6*).

The pattern of gene deletions varied by *BCR-ABL1* status with a higher frequency of *IKZF1* deletion in *BCR-ABL1*-positive ALL, as previously described,[24] and a higher frequency of *ETV6* and *RB1* deletions in *BCR-ABL1*-negative

Table 1. Clinical and outcome data for all B-other patients with gene rearrangements detected by fluorescence *in situ* hybridization or multiplex ligation-dependent probe amplification.

Patient ID	Trial	Abnormality	WCC (x10^9/L)	Outcome
25130	UKALL14	*IGH-CRLF2*	33.6	Died after 1 month
25371	UKALL14	*IGH-CRLF2*	47.7	Alive >5 years
28235	UKALL60	*IGH-CRLF2*	5.3	Relapsed and died after 2 years
30102	UKALL60	*IGH-CRLF2*	Not known	Relapsed and died after 5 months
30299	UKALL60	*IGH-CRLF2*	Not known	Died after 4 months
25246	UKALL14	*P2RY8-CRLF2*	6.3	Died within 1 month
28039	UKALL60	*P2RY8-CRLF2*	Not known	Died after 9 months
25552	UKALL14	*P2RY8-CRLF2*	2.9	Died after 4 months
28011	UKALL14	*P2RY8-CRLF2*	3.5	Died after 16 months
30297	UKALL60	*CRLF2-r*	Not known	Alive >2 years
30487	UKALL14	*IGH-CEBPA*	11.7	Alive after 1 year
25894	UKALL60	*IGH-CEBPD*	0.8	Alive >5 years
27181	UKALL14	*IGH-CEBPE*	1.2	Died after 3 months
27833	UKALL60	*IGH-BCL2*	14.5	Died after 2 years
25907	UKALL60	*IGH-r*	2	Died after 1 year
29808	UKALL60	*IGH-r*	Not known	Relapsed and died after 1 year
25451	UKALL14	*EP300-ZNF384*	34.2	Relapsed and died >5 years
25235	UKALL14	*ZNF384-r*	3.5	Alive >5 years
30085	UKALL60	*ZNF384-r*	Not known	Alive >2 years
25267	UKALL14	*MEF2D-r*	1.4	Alive >5 years

Outcome of patients with *CRLF2* rearrangement was very poor with only 2/10 alive 2 years after diagnosis. In comparison, 2/3 patients with *ZNF384* rearrangements were still alive after 5 years with only one relapse that occurred nearly 7 years after diagnosis. ID: identifier; WCC: white blood cell count.



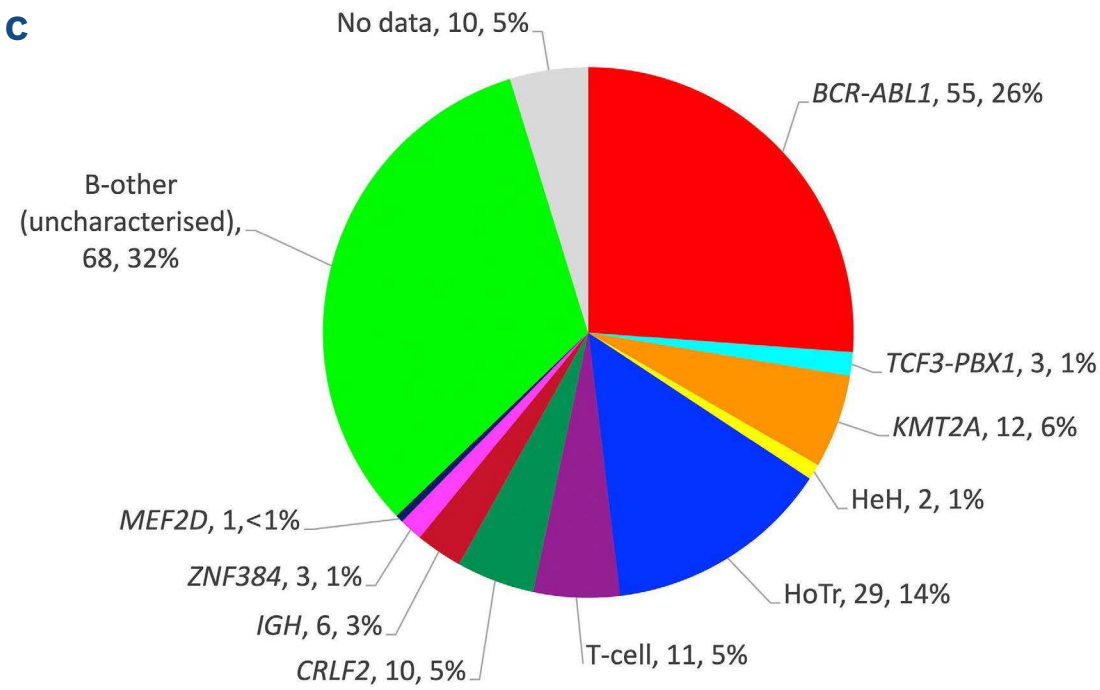


Figure 1. Distribution of primary chromosomal abnormalities by age groups across 210 adults aged ≥60 years. Primary genetic abnormalities shown by 5-year age groups, displayed by number (A) and proportion of cases (B) in each age group. Frequency of final genetic subgroups is shown following screening of B-other acute lymphoblastic leukemia cases for gene rearrangements (C). HoTr: low hypodiploidy/near triploidy; HeH: high hyperdiploidy; T-cell: T-cell acute lymphoblastic leukemia; B-other: B-cell precursor acute lymphoblastic leukemia in which no primary chromosomal abnormality was identified.

ALL (Table 2). Most deletions were heterozygous with the exception of *CDKN2A/B* which were homozygous in 50% of cases.

In total, 23% (18/78) of patients had no deletions in driver genes, 18% (14/78) had one deletion, 18% (14/78) had two deletions, 23% (18/78) had three deletions and 18% (14/78) had four or more gene deletions (*Online Supplementary Figure S1*). *IKZF1* deletions in particular co-occurred with other gene deletions much more commonly than in isolation (46% *vs*. 5%). The *IKZF1*^{plus} profile[17] was present in 36% (28/77) of the BCP-ALL samples, specifically in *BCR-ABL1*-positive (n=13), B-other (n=8) and HoTr (n=7) patients.

We detected recurrent focal CNA in several other genes, which to date have not been defined in the pathogenesis of ALL. Focal deletions in *LEMD3* on 12q14.3 and *KDM6A* on Xp11.3 were seen in 6% (5/78) and 5% (4/78) of cases, respectively. Demographic, genetic and outcome features of affected cases are shown in Table 3. *LEMD3* deletions ranged from 11-32kb in size (*Online Supplementary Table S7* and *Online Supplementary Figure S2*), although they were confined to intron 2 of the gene in three of these. *KDM6A* deletions ranged from 56-316 kb in size and were homozygous or hemizygous in three of the four cases (*Online Supplementary Table S7*). Deletion breakpoints for all *LEMD3* and *KDM6A* deletions were visually confirmed in IGV in the cases analyzed by NGS.

Mutational landscape

Twenty-three patients' samples covering all major genetic subgroups (*Online Supplementary Table S3*) were successfully sequenced using the 44-gene targeted panel. Across these samples, 25 single nucleotide variants and eight in-

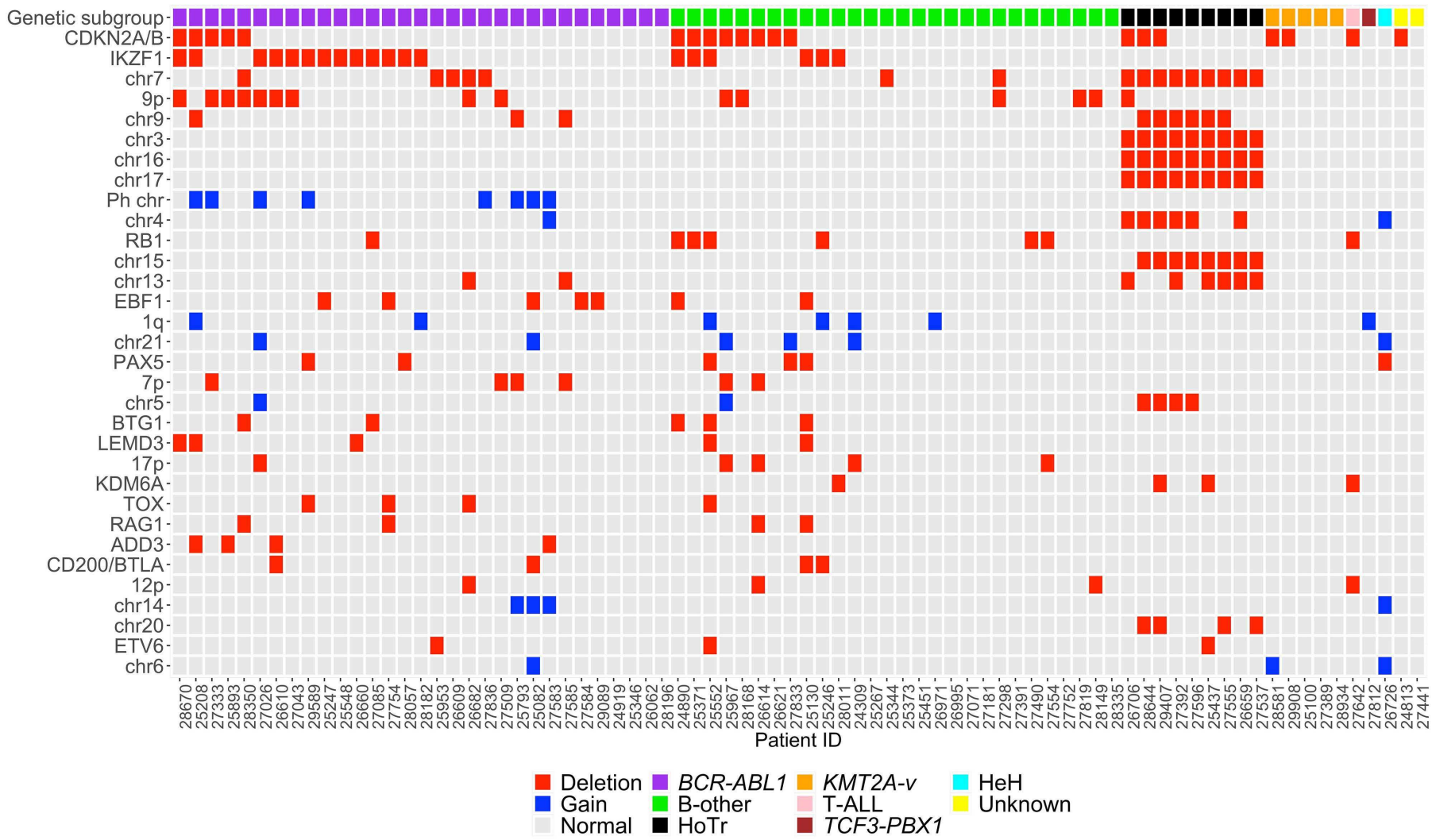


Figure 2. Complete copy number profile based on single nucleotide polymorphism array results from all 78 patients included in the single nucleotide polymorphism array cohort. Only copy number alterations present in at least three patients' samples are displayed. HoTr: low hypodiploidy/near triploidy; HeH: high hyperdiploidy; T-ALL: T-cell acute lymphoblastic leukemia; B-other: B-cell precursor acute lymphoblastic leukemia in which no primary chromosomal abnormality was identified.

Table 2. Frequency of individual deletions in known driver genes split by *BCR-ABL1* status. Significant differences identified in rate of *IKZF1*, *ETV6* and *RB1* deletions between *BCR-ABL1*-positive (*BCR-ABL*$^+$) and *BCR-ABL1* negative (*BCR-ABL1*$^-$) cases.

		Deletion frequency by *BCR-ABL1* status				
Gene	**Cases with deletion (n)**	***BCR-ABL1*$^+$ cases (n=31)**	***BCR-ABL1*$^-$ cases (n=47)**	***P*-value**	**Heterozygous deletions**	**Homozygous deletions**
IKZF1	51% (40)	68% (21)	40% (19)	0.02	41	2
CDKN2A	46% (36)	39% (12)	51% (24)	0.36	18	18
CDKN2B	46% (36)	39% (12)	51% (24)	0.36	21	15
PAX5	41% (32)	48% (15)	36% (17)	0.35	32	0
RB1	23% (18)	10% (3)	32% (15)	0.03	17	1
ETV6	21% (16)	6% (2)	30% (14)	0.02	16	0
EBF1	21% (16)	19% (6)	21% (10)	1	15	1
BTG1	13% (10)	6% (2)	17% (8)	0.3	9	1

dels were identified (Figure 3). At least one gene in the NGS panel was mutated in 74% (17/23) of patients. Pathogenic mutations in the RAS signaling pathway were identified in 17% (4/23) of cases. *KRAS* p.G12D and *KRAS* p.R68W variants were seen in patients with *KMT2A* and *EP300-ZNF384* rearrangements, respectively. *NRAS* p.G12S and p.G12D were present in one B-other case and one patient with unidentified genetic subgroup, respectively. With the exception of the *KRAS* p.R68W variant, all RAS pathway mutations are reported in the COSMIC database.[25]

Two patients' samples with HoTr were included. Consistent with the underlying chromosomal abnormality, a pathogenic *TP53* variant (*TP53* p.R282W) was detected in one of these.[26,27] Two indels in *NF1* and an *FLT3* missense variant (*FLT3* p.V194M) were also seen, the latter being reported as a tolerated passenger mutation in acute myeloid leukemia.[28]

Known pathogenic variants were also seen in *JAK2* (*JAK2* p.R683T in a patient with *IGH-CRLF2*), *CREBBP* (*CREBBP* p.L1499Q in a patient with *IGH-BCL2*), and *CSF1R* (*CSF1R*

p.V32G in a patient with *P2RY8-CRLF2*). Additionally, previously unreported *KDM6A* mutations were discovered in two *BCR-ABL1*-positive patients (*KDM6A* p.Y215H and p.K987Q).

We also investigated whether mutations associated with clonal hematopoiesis of indeterminate potential (CHIP) (most commonly affecting *DNMT3A*, *TET2* and *ASXL1*) were present in older adults with ALL.[29,30] These are found in 10% of adults over the age of 65 years without hematologic diseases, but are associated with an increased risk of subsequently developing myelodysplastic syndrome or acute myeloid leukemia.[30] Overall, these were discovered

Table 3. Demographic, clinical and outcome data of all cases with focal *LEMD3* or *KDM6A* deletions.

Gene	Patient ID	Sex (M/F)	Age (years)	Genetic subgroup	WCC (x10^9/L)	Outcome
LEMD3	25208	M	62	*BCR-ABL1*	205.4	Alive after 9 years
LEMD3	25130	F	62	*IGH-CRLF2*	33.6	Died after 1 month
LEMD3	28670	F	61	*BCR-ABL1*	1.6	Died after 2 months
LEMD3	26660	F	62	*BCR-ABL1*	18.2	Alive after 7 years
LEMD3	25552	M	61	*P2RY8-CRLF2*	2.9	Died after 4 months
KDM6A	28011	M	61	*B-other*	3.5	Died after 16 months
KDM6A	29407	F	60	*HoTr*	2.9	Died after 5 months
KDM6A	25437	F	64	*HoTr*	1.4	Died after 14 months
KDM6A	27642	F	72	*T-ALL*	Not known	Died after 18 months

All patients with *KDM6A* deletions died within 18 months of diagnosis. ID: identifier; M: male; F: female; WCC: white blood cell count; HoTr: low hypodiploidy/near triploidy; T-ALL: T-cell acute lymphoblastic leukemia

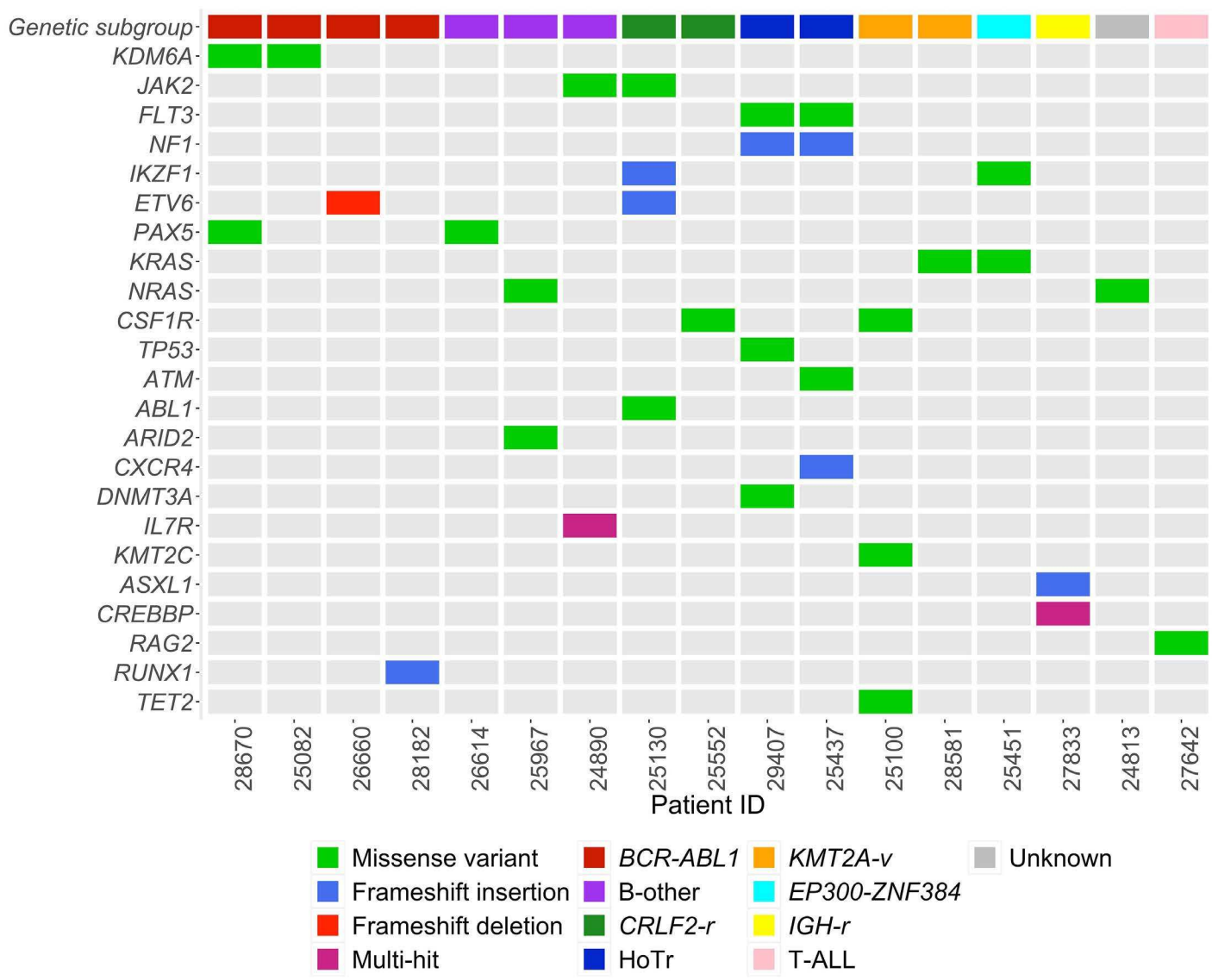


Figure 3. Mutations detected by the 44-gene next-generation sequencing panel in 23 patients. Only patients' samples with at least one mutation are displayed (n=17). In total, 24 single nucleotide variants, seven frameshift insertions and one frameshift deletion were identified. Two genes had both single nucleotide variants and indels within the same case ("multi-hit"). HoTr: low hypodiploidy/near triploidy; T-ALL: T-cell acute lymphoblastic leukemia; B-other: B-cell precursor acute lymphoblastic leukemia in which no primary chromosomal abnormality was identified.

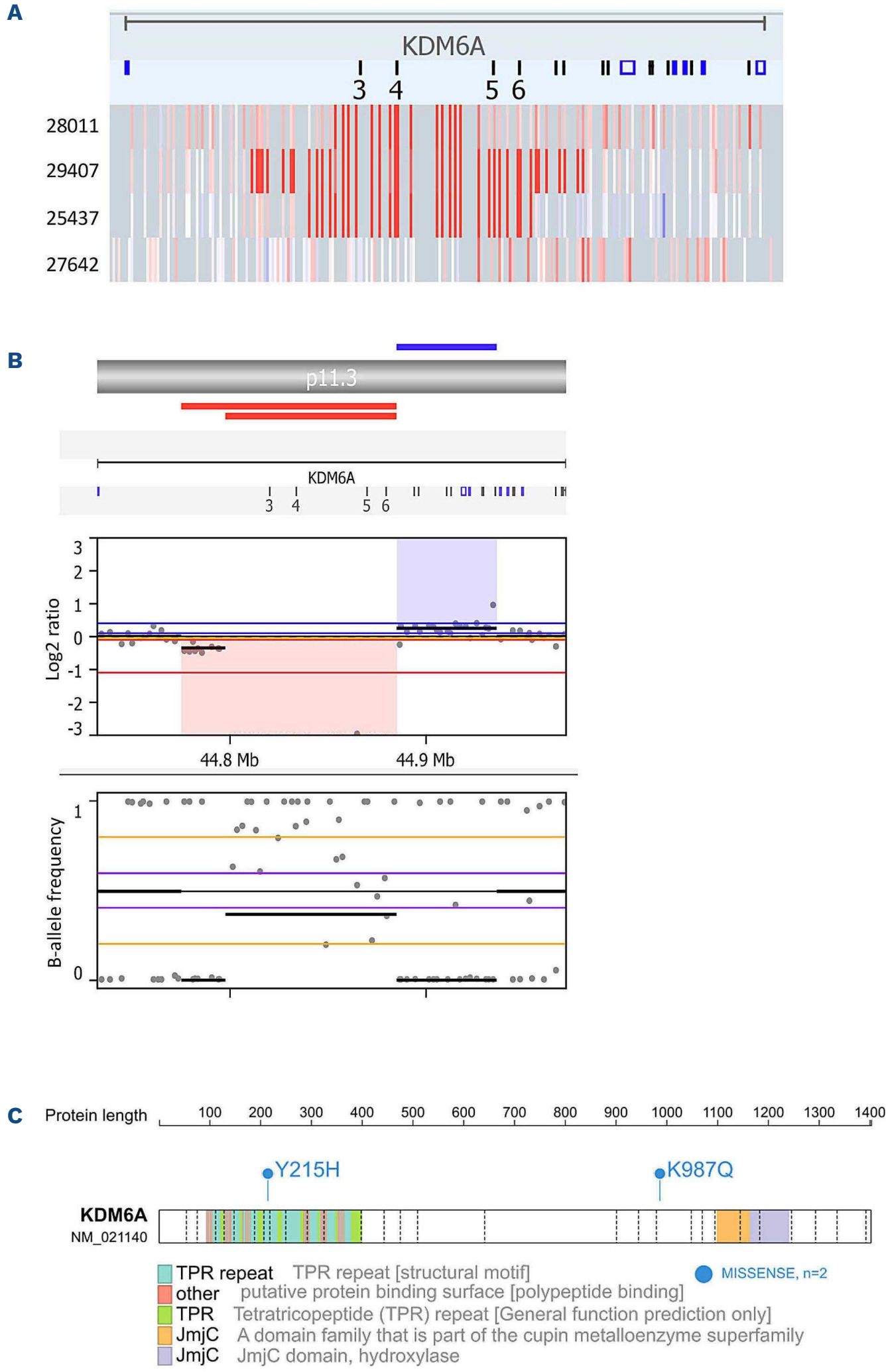


Figure 4. *KDM6A* aberrations detected by single nucleotide polymorphism array and next-generation sequencing. *KDM6A* deletions identified by single nucleotide polymorphism (SNP) array in four patients' samples (A). Each bar represents a probe on the SNP array. Red colors indicate negative $\log_2$ ratio (copy number loss), blue colors represent positive $\log_2$ ratio (copy number gain), and white represents no copy number change. Homozygous *KDM6A* deletion in patient 25437, demonstrating two slightly distinct *KDM6A* deletions measuring 110 kb and 87 kb, and resulting in biallelic loss of exons 3-6 (B). Small gain also noted following segment of homozygous deletion. *KDM6A* protein plot displaying two mutations detected by next-generation sequencing (C).

in only 13% (3/23) of cases with single variants in each of *DNMT3A*, *TET2* and *ASXL1*.

KDM6A alterations

Overall *KDM6A* was disrupted in six cases, with focal deletions in 5% (4/78) of SNP array samples (Table 3) and mutations in 9% (2/23) of NGS samples (Figure 3). Interestingly, the deletions resulted in homozygous or hemizygous *KDM6A* loss in three of the four cases (Figure 4A, *Online Supplementary Table S7*). Biallelic *KDM6A* deletions were seen in the two female patients with HoTr ALL, albeit by two different mechanisms. By cytogenetics and SNP array, patient #29407 had lost one copy of chromosome X and had a focal *KDM6A* deletion in the remaining homologue. In comparison, patient #25437 had two focal but subtly distinct intragenic *KDM6A* microdeletions on each X chromosome (Figure 4B). As *KDM6A* is not in a pseudoautosomal region, the male patient (#28011) had a deletion affecting the only *KDM6A* allele, resulting in hemizygous loss. The *KDM6A* mutations detected by NGS were present in exons 8 (*KDM6A* p.Y215H) and 20 (*KDM6A* p.K987Q) (Figure 5C) and are not reported in the literature although the SIFT[31] and Polyphen[32] *in silico* prediction tools describe deleterious and probably damaging consequences, respectively, consistent with loss of function. Patients with *KDM6A* deletions had a poor outcome and all four affected patients died 5-18 months after diagnosis (Table 2). Similarly, the two patients with *KDM6A* mutations both died within 2 months of diagnosis.

Patients' outcome by genetic subtype

Outcome data were available for analysis for all 95 UKALL14 patients. Five-year event-free survival and overall survival rates were 17% and 24%, respectively (Figure 5,

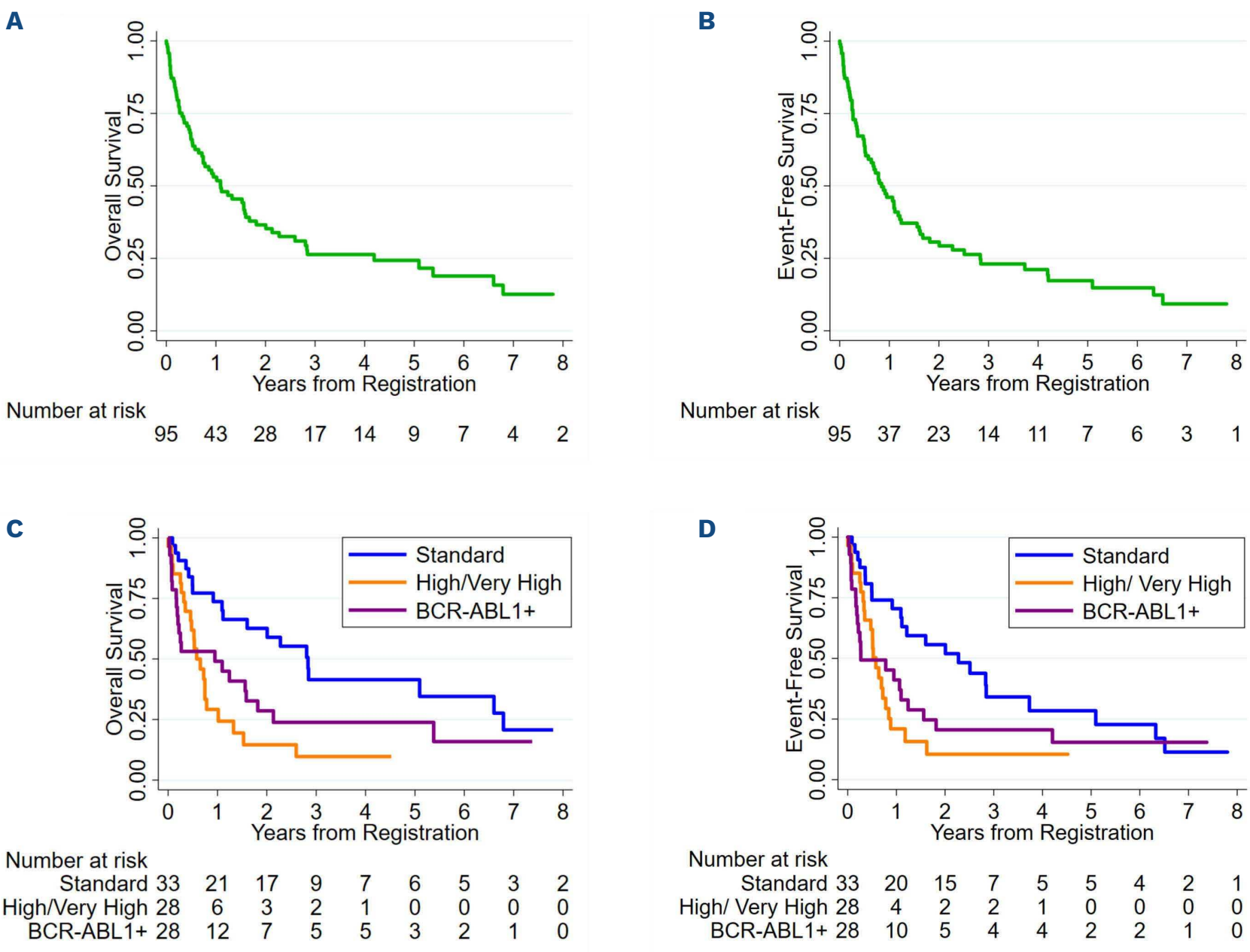


Figure 5. Overall survival and event-free survival for 95 adults aged ≥60 years recruited into the UKALL14 study. Overall survival (A) and event-free survival (B) for all patients combined; overall survival (C) and event-free survival (D) split by genetic risk group with very high risk and high risk combined into a single group. Patients with complex karyotypes, low hypodiploidy/near triploidy or *CRLF2* rearrangements were classed as very high risk; patients with any *KMT2A* fusions were classed as high risk; patients with *BCR-ABL1* and other kinase-activating fusions were classed as tyrosine kinase-activating abnormalities (all *BCR-ABL1*-positive in this study); all other patients with B-cell precursor acute lymphoblastic leukemia were classed as standard risk (SR). Patients with SR or *BCR-ABL1* had improved overall survival (P=0.001) and event-free survival (P=0.002) compared to patients with high or very high risk disease.

Online Supplementary Table S8). Even though the outcome of these older patients was poor there was evidence that tumor genetics remained a strong risk factor (Figure 5), as we have previously demonstrated for younger adults.[23] Patients with standard-risk genetics had the best outcome with 84% entering remission and 5-year event-free and overall survival rates of 28% and 41%, respectively. In comparison, over a third of patients with very high-risk genetics did not go into remission and all 28 patients with genetic high-risk or very high-risk disease died within 5 years of diagnosis.

Regarding patients with B-other ALL with gene rearrangements identified, all three patients with *ZNF384*-rearranged ALL survived more than 2 years from diagnosis whereas six out of eight of those with *CRLF2* rearrangements and four out of six with *IGH* translocations (excluding *CRLF2* partners) died within 2 years (Table 1).

Of the 40 patients with *IKZF1* deletions, no significant difference in outcome was identified between those with focal deletions of exons 4-7 (generating the dominant negative IK6 isoform)[33] compared with patients with other *IKZF1* deletions (*Online Supplementary Table S9*).

Discussion

To our knowledge, our study provides the largest genetic and genomic characterization to date of older adults with ALL.

The landscape of primary chromosomal abnormalities and CNA is distinct from that observed in children and younger adults. T-ALL was only seen in 5% of cases, which is less than half the rate seen in younger adults.[34]

Overall, a quarter of patients had *BCR-ABL1*-positive ALL, although the frequency of this did not increase further over the age of 60 years. This corroborates the findings from a large analysis of three German Multicenter study group for adult ALL (GMALL) trials in which the proportion of *BCR-ABL1*-positive cases reached a plateau after 45 years of age.[35] In comparison, our study highlights that HoTr becomes more common with advancing age, such that it is encountered in <2% of childhood patients,[36] 4-9% of adults aged 25-60,[23] rising to around 15% of adults aged 60 years and over in our cohort. Other high-risk cytogenetic subgroups, specifically *KMT2A* fusions and complex karyotypes were present in 6% and 3% of patients, respectively, which are similar rates to those seen in younger adults.[5,23] We found a lower frequency of *CRLF2* rearrangements than found in two USA studies that included older adults.[12,37] This could be related to the higher prevalence of Hispanic ancestry in the USA, and the associated inheritance of *GATA3* risk alleles, which confer an elevated risk of *CRLF2*-rearranged ALL.[38] In comparison to younger patients, ABL-class fusions were notably absent in our cohort. Other studies have similarly identified very low frequencies of ABL-class fusions in older individuals. Indeed, only two cases were identified in 103 adults aged 60 years or over in a recent large USA study.[6] Data from the GMALL group similarly demonstrated a rapid decrease in the frequency of Ph-like ALL in older age groups.[39]

Although we limited our survival analysis to UKALL14 patients, we highlight that prognosis remains very poor in older adults with ALL (Figure 5). However, consistent with recent studies, patients with *ZNF384* rearrangements seemed to have a favorable outcome.[23]

CNA in key genes recurrently disrupted in ALL were discovered in the majority of patients. *IKZF1* loss was present in over half of all cases tested by SNP array, occurring in 68% of *BCR-ABL1*-positive and 40% of *BCR-ABL1*-negative ALL. The high rate of *IKZF1* loss in *BCR-ABL1*-positive ALL is consistent with much of the published literature.[24,40] However, the frequency of *IKZF1* deletion in the older patients with *BCR-ABL1*-negative ALL was double that reported in younger adults (40% *vs*. 19%).[41] This discrepancy is at least in part driven by the increased frequency of low hypodiploid cases, as these usually only retain one copy of chromosome 7. However, *IKZF1* deletions were still encountered in 36% (10/28) of B-other cases. Deletions in other key driver genes in BCP-ALL (*CDKN2A/B*, *PAX5*, *RB1*, *ETV6* and *EBF1*) were also encountered more frequently than in younger patients.[23]

The high-risk *IKZF1*^{plus} copy number profile was identified in over a third of patients, although its prognostic impact in older adults still needs to be elucidated. Interestingly, no focal *ERG* deletions, which are associated with a favorable outcome, were detected.[42] Overall, these data confirm that all genetic biomarkers typically associated with a good prognosis, namely *ETV6-RUNX1* fusion, high hyperdiploidy and *ERG* deletions are exceedingly rare in older adults with ALL, contributing to the adverse outcomes of this population of patients. By virtue of the techniques used, we recognize that we were not able to identify certain novel subgroups, such as the recently described PAX5-driven subtypes[6] or *DUX4* rearrangements, although the latter are associated with *ERG* deletions in the majority of cases.[43]

Importantly, our study highlighted therapeutically actionable targets that would merit further investigation in older adults. We identified *KDM6A* deletions and mutations in 5% (4/78) and 9% (2/23) of screened patients, respectively. *KDM6A* (also known as *UTX*) on Xp11.3 is an H3K27me3 demethylase, involved in epigenetic regulation through repression of PRC2/EZH2 activity. Recurrent *KDM6A* mutations have been identified in T-ALL, and have been shown to have gender-specific tumor suppressor effects.[44] *KDM6A* escapes X-inactivation in females and therefore retains biallelic expression. Hence, loss of tumor sup-

pressor function through *KDM6A* abnormalities disproportionately affects males and has been postulated to explain the skewed gender distribution of T-ALL. To our knowledge, our study is the first to demonstrate *KDM6A* disruption in a significant proportion of older adults with ALL, most of whom had B-cell disease. Most interestingly, we have highlighted homozygous *KDM6A* deletions in female patients, and no evidence of skewed gender distribution. Loss of KDM6A function, resulting in EZH2 overactivity, has been shown to play an important pathogenic role in urothelial bladder cancer[45] and functional analyses have demonstrated susceptibility of KDM6A-null cell lines to the Food and Drug Administration-approved EZH2 inhibitor tazemetostat.[45] Our findings therefore identify a proportion of patients who may respond to EZH2 inhibition, a treatment as yet untested in ALL.

We also discovered recurrent small focal intragenic deletions in *LEMD3* in 6% (5/78) of cases. *LEMD3* regulates bone morphogenic protein and transforming growth factor β signaling and to date has not been implicated in cancer.[46] The significance of these deletions remains speculative, particularly as some were confined to introns.

Recurrent RAS pathway mutations were identified in almost one fifth of patients (4/23), all of whom had *BCR-ABL1*-negative ALL. These activate RAS signaling and are potentially therapeutically actionable through MEK inhibition (e.g., with selumetinib).

Older adults with ALL fare extremely poorly with current chemotherapeutic approaches. Various studies have demonstrated the disproportionate treatment toxicities experienced by this group of patients, leading to treatment omissions or delays.[20] Our analysis confirms the additional challenges posed by the high proportion of poor-risk genetic subgroups. Moving forward, the comprehensive identification of druggable targets such as *KDM6A* abnormalities, JAK-STAT-activating rearrangements or RAS pathway mutations presents an opportunity to expand therapeutic options, likely to most benefit this patient population. As a paradigm, significant progress has been made in the management of *BCR-ABL1*-positive disease through these approaches, culminating in a promising chemotherapy-free protocol.[47] Further dedicated clinical trials that include comprehensive genomic profiling of older adults, combined with targeted treatments and/or immunotherapy and a reduction in the traditional chemotherapy backbone will be key to improving the dismal outcome of these patients.

Disclosures

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Contributions

TC and AVM designed the study. TC, AVM, EB and EB collected and assembled the data. TC, AVM, EB, EB and SLR performed data analysis and interpretation. CJH and AVM were responsible for administrative support. AKF was chief investigator of the UKALL14 and UKALL60+ clinical trials. AAK, DL, EP, PP, LCH, BP, TM, AKM, CJR, NM, DIM, and AKF participated in recruitment of patients and provided study materials. TC and AVM developed the first drafts of the manuscript. All authors contributed to the review and amendments of the manuscript and approved the final version for submission.

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Data-sharing statement

The datasets generated or analyzed during the current study are available in the Online Supplementary Material or from the corresponding author on reasonable request.

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