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Molecular Classification Improves Risk Assessment in Adult *BCR-ABL1*-negative B-ALL

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Abstract:
Genomic classification has improved risk assignment of pediatric but not adult B-lineage acute lymphoblastic leukemia (B-ALL). The international UKALLXII/ECOG-ACRIN E2993 (NCT00002514) trial accrued 1229 *BCR-ABL1*-negative adolescent/adult B-ALL patients (aged 14-65 years). While 93% of patients achieved remission, 41% relapsed at a median of 13 months (range 28 days to 12 years). Five-year overall survival (5yr-OS) was 42% (95% CI, 39, 44). Transcriptome sequencing (n=238), gene expression profiling (n=210), cytogenetics (n=197) and fusion PCR (n=274) enabled genomic subtyping of 282 patient samples, of which 264 were eligible for trial, accounting for 64.5% of E2993 patients. Among patients in the outcome analysis, 29.5% of cases had favorable outcomes with 5yr-OS of 65-80% and were deemed standard-risk (*DUX4*-rearranged [9.2%], *ETV6-RUNX1/-like* [2.3%], *TCF3-PBX1* [6.9%], PAX5 P80R [4.1%], high-hyperdiploid [6.9%]); 50.2% had high-risk genotypes with 5yr-OS of 0-27% (Ph-like [21.2%], *KMT2A-AFF1* [12%], low-hypodiploid/near-haploid [14.3%], *BCL2/MYC*-rearranged [2.8%]); and 20.3% had intermediate-risk genotypes with 5yr-OS of 33-45% (PAX5alt [12.4%], *ZNF384/-like* [5.1%], *MEF2D*-rearranged [2.8%]). *IKZF1* alterations occurred in 86% of Ph-like and TP53 mutations occurred in low-hypodiploid (54%) and *BCL2/MYC*-rearranged patients (33%), but were not independently associated with outcome. Of patients considered high-risk for relapse based on presenting age and WBC count, 40% harbored subtype-defining genetic alterations associated with standard- or intermediate-risk outcomes. We identified distinct immunophenotypic features for *DUX4*-rearranged, PAX5 P80R, *ZNF384*-R/-like and Ph-like genotypes. These data in a large adult B-ALL cohort treated with a non-risk-adapted approach on a single trial show the prognostic importance of genomic analyses which may translate into future therapeutic benefits.

Conflict of interest: COI declared - see note

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Short Title: Genomic classification improves outcome in B-lineage ALL

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Key Points:

- Genomic testing leads to improved risk assignment in the majority of adult patients with *BCR-ABL1*-negative B-lineage ALL.
- Antigen profiles affect outcome in B-ALL when considered in conjunction with genomic subtype.

Abstract

Genomic classification has improved risk assignment of pediatric but not adult B-lineage acute lymphoblastic leukemia (B-ALL). The international UKALLXII/ECOG-ACRIN E2993 (NCT00002514) trial accrued 1229 *BCR-ABL1*-negative adolescent/adult B-ALL patients (aged 14-65 years). While 93% of patients achieved remission, 41% relapsed at a median of 13 months (range 28 days to 12 years). Five-year overall survival (5yr-OS) was 42% (95% CI, 39, 44). Transcriptome sequencing (n=238), gene expression profiling (n=210), cytogenetics (n=197) and fusion PCR (n=274) enabled genomic subtyping of 282 patient samples, of which 264 were eligible for trial, accounting for 64.5% of E2993 patients. Among patients in the outcome analysis, 29.5% of cases had favorable outcomes with 5yr-OS of 65-80% and were deemed standard-risk (*DUX4*-rearranged [9.2%], *ETV6-RUNX1/-like* [2.3%], *TCF3-PBX1* [6.9%], PAX5 P80R [4.1%], high-hyperdiploid [6.9%]); 50.2% had high-risk genotypes with 5yr-OS of 0-27% (Ph-like [21.2%], *KMT2A-AFF1* [12%], low-hypodiploid/near-haploid [14.3%], *BCL2/MYC*-rearranged [2.8%]); and 20.3% had intermediate-risk genotypes with 5yr-OS of 33-45% (PAX5alt [12.4%], *ZNF384/-like* [5.1%], *MEF2D*-rearranged [2.8%]). *IKZF1* alterations occurred in 86% of Ph-like and TP53 mutations occurred in low-hypodiploid (54%) and *BCL2/MYC*-rearranged patients (33%), but were not independently associated with outcome. Of patients considered high-risk for relapse based on presenting age and WBC count, 40% harbored subtype-defining genetic alterations associated with standard- or intermediate-risk outcomes. We identified distinct immunophenotypic features for *DUX4*-rearranged, PAX5 P80R, *ZNF384*-R/-

like and Ph-like genotypes. These data in a large adult B-ALL cohort treated with a non-risk-adapted approach on a single trial show the prognostic importance of genomic analyses which may translate into future therapeutic benefits.

Introduction

Despite recent advances in therapeutic options for patients with B-lineage acute lymphoblastic leukemia (B-ALL), treatment of adults remains challenging because of a high incidence of adverse prognostic factors and poorer tolerance of intensive therapy. The Medical Research Council (MRC), now National Cancer Research Institute, UKALLXII/ECOG-ACRIN Cancer Research Group E2993 trial (ClinicalTrials.gov NCT00002514) accrued 1229 adult patients with *BCR-ABL1*-negative B-ALL between 1993 and 2006.[1-3] Among these patients, 41% of cases with successful cytogenetics had recurrent genetic abnormalities: t(v;11q23*)* (rearrangement of *KMT2A*/*MLL*), t(12;21)(p13;q22) (*ETV6-RUNX1*), t(1;19)(q23;p13.3) (*TCF3-PBX1*), t(5;14)(q31;q32) (*IGH-IL3*), or aneuploidy.[4] Thus, in adult *BCR-ABL1*-negative B-ALL, the majority of patients historically fell into the category of B-ALL not otherwise specified.[5]

Incorporation of measurable residual disease (MRD) to improve risk stratification, use of pediatric-inspired regimens for young adults, and the advent of targeted therapies, including monoclonal antibodies and chimeric antigen-receptor T-cells, have redefined the therapeutic paradigm of ALL.[6] The genomic revolution has also led to improved categorization of B-ALL.[7-10] In particular, the Ph-like (*BCR-ABL1*-like) phenotype, characterized by a gene-expression profile similar to

BCR-ABL1 positive ALL (*BCR-ABL1* ALL*)* but with non-*BCR-ABL1* genetic alterations activating kinase signaling or alterations of lymphoid transcription factor genes,[11-14] has poor prognosis in both children and adults.[15-20] Due to their prognostic impact, Ph-like and hypodiploid B-ALL have been added as a provisional entities to the WHO Classification of ALL.[21,22]

In light of these advances, we explored the clinical outcomes of the largest cohort ever reported of adult *BCR-ABL1*-negative B-ALL, who were treated without any risk-adapted modifications, on the UKALLXII/E2993 trial. In parallel, we performed genomic analysis of E2993 patients to gain insight into associations of novel genotypes with treatment response.

Methods

Patient identification and treatment design

The MRC enrolled 820 and ECOG-ACRIN 409 *BCR-ABL1*-negative B-ALL patients. Immunophenotyping and PCR for known fusions were performed centrally for E2993 patients (supplemental materials), while the MRC centrally reviewed institutional immunophenotyping. Local cytogenetic data were reviewed centrally.[4] Chemotherapeutic regimens of the trial have been described.[1-3,23] Patients over 35 years of age or with a WBC count greater than $30x10^9$/L at presentation were designated high-risk. The Institutional Review Board of participating centers approved this study, and informed consent was obtained in accordance with the Declaration of Helsinki.

Genomic profiling

Transcriptome sequencing (RNA-seq) was performed on 238 samples using TruSeq library preparation and HiSeq 2000/2500 or NovaSeq 6000 sequencers (Illumina). All sequence reads were paired-end, and sequencing was performed using (1) total RNA and stranded RNA-seq [100 base-pair (bp) reads] or (2) polyA-selected mRNA (100bp reads). Methods for RNA-seq processing and analysis are detailed in the supplemental materials. In a complementary approach, a 15-gene Taqman® quantitative RT-PCR low density array (LDA)[15,24,25] that identifies the Ph-like ALL gene signature, *P2RY8-CRLF2*, *BCR-ABL1*, *ETV6-RUNX1*, *TCF3-PBX1*, *KMT2A*-rearranged and *DUX4*-rearranged ALL was performed on 210 samples.

To determine copy number alterations (CNAs) including focal deletions, single nucleotide polymorphism (SNP) analysis was performed on 129 of 282 samples with available DNA using either SNP 6·0 microarrays (Affymetrix, n=47) or the Infinium Omni2.5Exome-8 BeadChip Kit (Illumina, n=82) as previously described.[14,15] DNA and RNA-seq data was available for 102 samples using the FoundationOne Heme panel.[26] PCR and Sanger Sequencing for single nucleotide variants (SNVs) and insertion/deletions (indels) was available for 47 samples.[14] Genomic data is publicly available and has been deposited in the European Genome Phenome Archive, Accessions EGAS00001000654, EGAS00001001952, EGAS00001003266 and EGAS00001004638.

Molecular subtyping

Genomic classification for 282 cases (supplemental Figure S1) was based on the use of multiple assays where available: RNA-seq (n=238), LDA (n=210), cytogenetics (n=197) and PCR for known fusions (*ETV6-RUNX1*, *KMT2A-AFF1* and *TCF3-PBX1*; n=274) (supplemental Figure S2A). The overall schema for molecular classification of B-ALL, as described previously,[27] is provided in supplemental Figure S3. For cases with RNA-seq (n=238), we identified 17 leukemia subtypes defined by CNA for aneuploid cases, recurring genetic alterations (subtype-defining rearrangements and SNVs), distinct clustering by two-dimensional t-Distributed Stochastic Neighbor Embedding (tSNE) plot, and gene expression profiles determined by Prediction Analysis of Microarrays (PAM)[28] (supplemental Figure S4). For the remaining cases with cytogenetic and/or LDA data (n=43), we identified subtypes based on aneuploidy (hyperdiploidy, hypodiploidy), the presence of known fusions (*ETV6-RUNX1*, *KMT2A*-R, *TCF3-PBX1*) and gene expression (*DUX4*-R, Ph-like). One remaining case with *KMT2A-AFF1* was identified by routine clinical PCR. Details for the 282 E2993 cases with molecular classification are provided in supplemental Table S1.

Copy number alteration (CNA) detection

RNA-seq data may be used to robustly identify chromosomal and arm level CNAs (https://github.com/honzee/RNAseqCNV/). Genes were ordered based on the median absolute deviation (MAD) of their expression level across the samples and a subset (1/4 to 1/3) of the genes with least MAD were picked as stably expressed

genes. To assist the CNA calling, mutant allele frequency of SNVs detected from RNA-seq data were plotted against the gene expression level of the stably expressed genes to double check if the CNAs were reliable.

Statistical methods

Patient characteristics and remission rates were compared using χ^2 tests for heterogeneity, Mantel-Haenszel test for trend or the Mann-Whitney U test. The donor-no donor comparison included patients <50 or 55 years (age limits for potential ASCT). Censoring was at the earlier of date of last contact, or October 31, 2010, the data cut-off date. Relapse-free survival (RFS) was calculated from CR to relapse or death without relapse. Time to relapse was measured from start of induction. The log-rank method was used to compare time-to-event distributions. Where the hazard was nonproportional and the long-term survival was to be compared, the χ^2 *P* value for the difference in the survival percentages at 5 years was quoted. Cox regression analysis was used to investigate the association between a time-to-event variable and baseline covariates. Transplantation was entered into multivariable Cox models as a time-varying covariate. All *P* values were two-sided.

Both percent of leukemic lymphoblasts stained by individual antibodies and the median fluorescence intensity (MFI) of antibody binding divided by MFI in the absence of antibody (MFI ratio) were compared across all genetic subtypes by Kruskal-Wallis test, and then between all pairs of genetic subtypes by the Mann-Whitney U test, yielding 1501 comparisons. The P value threshold of 0.0209 was

determined by the adaptive profile information criteria[29] which balance the levels of false discovery and false negative rates.

Data Sharing

Genomic data is publicly available and has been deposited in the European Genome Phenome Archive, Accessions EGAS00001000654, EGAS00001001952, EGAS00001003266 and EGAS00001004638.

Results

Biologic features for 1229 B-ALL patients

The median age of the cohort was 30 years (range 14 to 65). The median WBC count at presentation was 9.2 x 10^9/L (range 0.1-930) and was <30 x 10^9/L in 73% of the patients (supplemental Table S2).

By immunophenotyping, 18.5% were CD10- pro-B ALL, 63% were CD10+ early pre-B ALL, 15.7% had pre-B ALL with cytoplasmic IgM and 2.8% were mature B-ALLs. No survival difference was seen according to maturational stages, when excluding mature B-ALL (supplemental Figure S5).

Cytogenetic analysis was unavailable in 401 (32.6%), normal (≥20 normal bone marrow metaphases) in 199 (24%) and abnormal in 629 patients (76%), based on standard G-banding (supplemental Table S3).

Molecular characterization, demographics and antigen expression in E2993 patients

Molecular analysis was possible for 282 E2993 patients, of whom 264 were eligible for trial (supplemental Figure S1). With the exception of older age (median 35 versus 29 years, p<0.001), this cohort did not differ with respect to demographic or clinical variables from *BCR-ABL1*-negative B-ALL UKALLXII/E2993 patients who were not tested (supplemental Table S4). Thus, our study cohort is representative of the entire trial population.

In addition to subtypes recognized by karyotyping or PCR for chimeric fusions, such as *ETV6-RUNX1*, *TCF3-PBX1*, *TCF3-HLF*, *KMT2A*-rearranged (*KMT2A*-R) and aneuploid ALL (high-hyperdiploid, HH; low-hypodiploid, LH; near-haploid, NH; intrachromosomal amplification of chromosome 21, iAMP21), recently described genotypes included *DUX4*-rearranged (*DUX4*-R),[30,31] *ETV6-RUNX1*-like,[32] PAX5alt and PAX5 P80R,[27,33-35] Ph-like with rearrangements and/or overexpression of *CRLF2* (Ph-like *CRLF2*-R) and Ph-like with genetic alterations activating other kinases or cytokine receptor signaling pathways (Ph-like non-*CRLF2*-R),[11-14] *ZNF384*-rearranged (*ZNF384*-R) and *ZNF384*-R-like[30,36-39], *MEF2D*-rearranged (*MEF2D*-R),[40,41] *BCL2/MYC*,[27] and the genotype with concomitant ZEB2 and CEBPE alterations,[34] using established criteria (supplemental Table S5 and supplemental Figure S3). For outcome analysis, LH and NH patients were combined despite their distinct biologies,[42,43] because of their comparably poor prognosis.[44] Cases without a subtype-defining driver genetic alteration were excluded from further analysis ("B-other") (11%). Demographics and frequencies of genomic subgroups are summarized in Table 1. Barriers to the enrollment of

minorities to adult cancer clinical trials during E2993, before current NIH initiatives,[45] prevented associations between genotype and race or ethnicity.

In 51% of cases, cytogenetic analysis failed or yielded a normal result. While for recurrent gene fusions, corresponding cytogenetic abnormalities were frequently detected, the majority of genetic alterations were cryptic in nature (supplemental Table S1). Within the Ph-like cohort, 62.5% had Ph-like *CRLF2*-R versus 37.5% with Ph-like non-*CRLF2*-R, similar to other reports in adult ALL.[15-18] Among Ph-like *CRLF2*-R cases, the ratio of *IGH-CRLF2* to *P2RY8-CRLF2* rearrangements was 3:1, supporting a prevalence of *IGH-CRLF2* in adults.[15,16] Genetic alterations in Ph-like non-*CRLF2*-R cases included rearrangements and mutations driving JAK-STAT signaling (*JAK2* [n=4]*, EPOR* [n=2], *IL7R* [n=1], *TYK2* [n=1]), fusions involving ABL-class genes (ABL1 [n=1], ABL2 [n=1], PDGFRA [n=1]), and one case with *ETV6-NTRK3* fusion. As observed in pediatric ALL,[32] a fusion involving *IKZF1* was found in one of our *ETV6-RUNX1*-like ALL patients. Among *DUX4*-R, *IGH-DUX4*[30-32] and among *MEF2D*-R patients, *MEF2D-BCL9* fusions were most common.[40,41,46] *ZNF384* was recurrently rearranged to the transcriptional regulator EP300,[36,38] while other partners (TAF15, TCF3)[37] were less common. All of the *BCL2/MYC* cases harbored rearrangements of *BCL2*, *BCL6* and/or *MYC* (Table 2).

The presence of SNVs, insertion/deletions (indels) and focal deletions was determined for key genes including *IKZF1*, *TP53*, and those related to JAK-STAT signaling (*JAK1*, *JAK2*, *IL7R*, *SH2B3*, *CRLF2*) (supplemental Figure 2B and supplemental Table S6). We observed a higher frequency of *IKZF1* alterations (deletion or mutation) in Ph-like than non Ph-like cases (27 of 31, 87% vs. 14 of

100, 14%), confirming earlier data.[14] Overall, alterations in the JAK-STAT signaling pathway were identified in 15 of 271 cases assessed (5.5%), predominantly in Ph-like ALL. Among 30 Ph-like *CRLF2*-R cases, we observed mutation of *JAK1* (n=1), *JAK2* (n=8) and *IL7R* (n=1). Two Ph-like *CRLF2*-R cases harbored CRLF2 p.Phe232Cys in addition to mutation of *JAK1* or *JAK2*. An additional *IL7R* indel was identified in a Ph-like non-*CRLF2*-R case. One PAX5 P80R case harbored two JAK2 mutations (p.Arg683Gly and p.Asp873Asn) and one B-other case with *IGH-CRLF2* (lacking the Ph-like signature) harbored CRLF2 p.Phe232Cys. *TP53* mutations were more common in LH (15 of 28, 54%), as expected,[42] and *BCL2/MYC*-R (4 of 12, 33%) compared to the remaining cohort (5 of 231, 2.2%).

Clustering of antigen expression for samples with defined genotypes is illustrated in Figure 1 and distinguishing immunophenotypic features are described in supplemental Table S7. Unique observations included overexpression of CD13, CD34 and CD38 by *DUX4*-R compared to other genotypes ($p<0.0001$). The T-cell antigen CD2, present in 68% of *DUX4*-R, was also found in 50% of PAX5 P80R patients. PAX5 P80R blasts were arrested at the pro-B stage, though with higher frequency of CD10+ blasts ($p=0.0011$) and stronger CD10 expression ($p=0.0052$) compared to prototype pro-B *KMT2A*-R blasts. PAX5 P80R blasts expressed CD33, while lacking $CD65_S$ and $CD15_S$, the two myeloid antigens typically[47] expressed jointly in *KMT2A*-R. Antigen expression was indistinguishable between *KMT2A-AFF1* and *KMT2A*-non-*AFF1* blasts. Ph-like blasts with or without *CRLF2*-R differed only by a higher frequency of CD13+ blasts in Ph-like non-*CRLF2*-R cases ($p=0.011$). Compared with *BCR-ABL1,* Ph-like blasts showed comparably weak

CD38 but less dual CD13-CD33 (p=0.0001) and CD25 expression (p=0.0003), both characteristic features of *BCR-ABL1* ALL.[48] *ZNF384*-R/-like blasts were CD10- in 73% of cases but compared to *KMT2A*-R, CD24+ (p=0.0088) and CD20+ (p<0.0001) blasts were more frequent. *ZNF384*-R/-like blasts lacked $CD65_S$ and $CD15_S$ but expressed dual CD13-CD33, comparable to *BCR-ABL1* ALL, and CD25 in one-third of cases. Myeloperoxidase was not detected. There was neither overexpression of surface CD135 nor evidence of FLT3-ITD, a potentially targetable protein kinase suggested for *ZNF384*-R.[49] Blasts with *BCL2/MYC*-R genotype showed features of mature B-ALL with surface immunoglobulin monoclonality and strong CD20 but variable TdT and CD10 expression. LH blasts were often CD10- and $CD65_S$+ or $CD15_S$+, though dual $CD65_S$-$CD15_S$ expression was rarer in LH than *KMT2A*-R cases (p<0.0001).

Outcome in the entire UKALLXII/E2993 B-ALL cohort

Of 1229 patients with *BCR-ABL1*-negative B-ALL, 93% achieved CR. The CR rate was higher in younger patients (p<0.0001), but did not differ by sex, WBC count or state of CNS involvement. In multivariate Cox analysis, patients with LH or complex karyotype or t(8;14) had inferior survival. Relapse occurred in 507 patients (41%). The median time to relapse was 13 months (range 28 days to 12 years). Among relapsed patients, 116/373 without transplant in first CR had a transplant post relapse (12 autologous, 30 sibling-ASCT, 66 matched-unrelated-donor and 8 alternative-donor transplants). Of the 109 relapsed patients who had an ASCT post

relapse, 42 survived for a median of 4.5 years (10 days to 14.9 years) from time of relapse.

The 5-year OS was 42% (95% CI, 39, 44). In multivariate Cox analysis considering age, sex, WBC count, CNS disease and interaction between CNS disease and WBC count, increasing age ($p<0.0001$), higher WBC count ($p<0.0001$) and CNS involvement at diagnosis ($p=0.02$) were associated with shorter OS. In 326 patients randomized between chemotherapy and autologous transplant, the 5-year OS was 47% (95% CI 39, 54) versus 35% (95% CI 28, 43) ($p=0.03$). In the donor-no donor analysis, the 5-year OS was 52% (95% CI 47, 58) for patients with a donor and 45% (95% CI 41, 50) for patients without (log rank $p>0.1$; χ^2 test of difference at 5 years, $p=0.05$) (Figure 2 with results carried out to 10 years). The relapse risk at 5 years for those with a donor was 30% (95% CI 24, 35) versus 55% (95% CI 50, 60) in those without (log rank $p<0.0001$; χ^2 at 5 years, $p<0.0001$). The percentage of deaths in remission at 5 years for those with a donor was 32% (95% CI 26, 37) versus 10% (95% CI 7, 13) for those without (log rank $p<0.0001$; χ^2 at 5 years, $p<0.0001$).

Outcome among molecular subgroups

Of 282 patients genotyped, 264 were treated on study. Outcome analysis was limited to subgroups with ≥5 patients and was done for 217 patients (see supplemental Figure S1 for patient selection). *KMT2A-non-AFF1* patients were excluded due to their molecular heterogeneity, though outcome did not differ from

that in *KMT2A-AFF1* patients. Ph-like patients with and without *CRLF2*-R were combined as their outcomes were superimposable.

CR rates ranged from 50% in the *BCL2/MYC* to 100% in the *ETV6-RUNX1*/-like, HH, PAX5 P80R and *MEF2D*-R groups (supplemental Table S8). Confidence intervals overlapped, possibly due to the small sizes of some of the cohorts.

With a median of 8 years and 9 months of follow-up for survivors, RFS (supplemental Table S9) and OS (supplemental Table S10) varied markedly among groups (Figure 3). In Cox proportional-hazards models, outcomes of all groups were compared to that of *DUX4*-R as reference group. In univariable analysis (Table 3), *DUX4*-R, HH, *ETV6-RUNX1*/-like, PAX5 P80R and *TCF3-PBX1* patients had comparable RFS and were designated molecular standard-risk (mSR). *BCL2/MYC*-R, *KMT2A-AFF1*, LN/NH and Ph-like patients did significantly worse than *DUX4*-R ($p<0.001$), and they were considered molecular high-risk (mHR). PAX5alt, *ZNF384*-R/-like and *MEF2D*-R patients had intermediate-risk (mIR) of relapse with RFS inferior to *DUX4*-R patients, though with lower levels of significance. OS among mSR groups did not vary significantly. OS of mHR groups remained inferior to that of *DUX4*-R ($p<0.001$), while among mIR, only PAX5alt patients remained significantly inferior to *DUX4*-R ($p=0.01$) but not *ZNF384*-R/-like ($p=0.111$) or *MEF2D*-R ($p=0.098$) (Table 3). Molecular analysis stratified 64 patients (29.5%) as mSR, 44 (20.3%) as mIR, and 109 (50.2%) as mHR. To consider an effect of antigens on outcome beyond their association with various genotypes, we adjusted multivariable Cox models for the presence of CD34, CD10, CD19, CD24,

CD20, CD22 and CD38. Neither antigen expression nor the type of transplant received affected prognosis (not shown).

When comparing outcome of *IKZF1*-altered (n=41) versus *IKZF1* wild-type (n=90) across the whole cohort, no difference was detected in either OS or RFS. This was also true when Ph-like status was included in multivariable analyses with or without an interaction term. Among Ph-like patients, OS did not differ by *IKZF1*-status, in part due to the expected[12,14,15] high frequency of *IKZF1* alterations in this group (25 of 29 cases treated on trial, 86%). With respect to RFS, cases with wild-type *IKZF1* fared much better than *IKZF1*-altered cases, though cohorts were too small to reach significance (supplemental Figure S6).

When applying the protocol-specified risk classification (age >35 years or WBC count >30x10^9/L) to 216 molecularly characterized patients, 137 were allocated to the protocol-defined high-risk group (pHR). Of pHR patients, 21.2% had mSR and 18.2% had mIR genotypes (supplemental Table S11). pHR patients with mSR or mIR genotypes did significantly better than mHR patients among the pHR cohort (for RFS, HR=0.36 [95% CI 0.23, 0.57], p< 0.001; for OS, HR=0.39 [95% CI 0.25, 0.60], p< 0.001) (supplemental Table S12).

Figure 4 shows the effect of protocol-defined low- (pLR) and high-risk (pHR) on RFS and OS in molecular risk groups. RFS was not different between protocol-defined risk groups within each molecular risk group, while OS was positively affected both for mSR (HR=0.24, p=0.007) and mHR patients (HR=0.47, p=0.007) when these patients fit the criteria for pLR. Superior 5-year RFS and OS was experienced by patients in the pLR/mSR cohort (73.3% [95% CI 59.8, 89.9] and

87.9% [95% CI 77.5, 99.8], respectively), while patients in the pHR/mHR cohort did worst (11.9% [95% CI 6.23, 22.9] and 12.9% [95% CI 7.19, 23.1], respectively) (Table 4).

As patients with sibling donor in the entire B-ALL cohort did better than patients without a donor (Figure 2), a donor-no donor analysis was done for molecular risk groups. Only 125 patients with molecular characterization had donor information available (57.6%). Neither RFS nor OS differed by donor availability in the mSR and mIR risk groups (supplemental Figure S7). In mHR patients, the data suggest longer RFS (p=0.08) and OS (p=0.2) for patients with a donor. Due to the small sample size of subgroups, interpretations of this data with respect to transplant recommendations are limited.

Discussion

A unique feature of our study is the integration of data from genomic testing and immunophenotyping with outcome in a large subset of unselected adult *BCR-ABL1* negative B-ALL patients treated on a single trial. Only 11% of patients were not assigned to a well-described genotype, a percentage as low as that achieved in children.

A review of prognostic biologic features in the entire UKALLXII/E2993 *BCR-ABL1*-negative B-lineage cohort revealed that cytogenetic anomalies, but not B-cell maturation stage of leukemic lymphoblasts, adversely affected outcome. However, standard chromosome analysis was non-informative in 50% of E2993 patients. Since most genotypes in E2993 were not associated with recurrent chromosomal

abnormalities (supplemental Table S1), cytogenetic analysis cannot be considered an adequate approach for their detection.

Among genotypes identified, Ph-like ALL constituted the biggest group (17%), comparable to most studies in adult ALL.[15,17,18,27] Although Ph-like and *BCR-ABL1* ALL have similar genetic profiles, the immunophenotype of Ph-like ALL has remained undefined.[50] We show here that their antigen expression is similar and distinctive from that in other genotypes with respect to weak CD38 expression, but also different with lower frequency of dual CD13-CD33 positive and CD25+ blasts in Ph-like ALL. We describe, for the first time, CD25+ CD10-/dim pro-B *ZNF384*-R/-like blasts. While overexpression of CD25 and its gene, interleukin-2 receptor α, are negative prognostic factors in *BCR-ABL1* ALL and *BCR-ABL1*-negative ALL,[51-53] CD25 did not affect outcome in *ZNF384*-R/-like patients (not shown). This supports the notion that antigen profiles affect outcome only when analyzed in conjunction with their underlying molecular counterparts. We found expression of the T-cell antigen CD2 not only in *DUX4*-R[54,55] but also in PAX5 P80R cases. CD2 expression in two novel genotypes with favorable outcome supports its proposed role as a surrogate for good prognosis in pediatric B-ALL in the early literature.[56-58]

As previously seen in adult ALL,[15-19,33,34,59] outcome of Ph-like patients was extremely poor, though we did not see inferior outcome of Ph-like *CRLF2*-R compared with Ph-like non-*CRLF2*-R patients.[16] Noteworthy, an analysis of UKALLXII patients with deregulated *CRLF2* expression showed reduced event-free survival and OS.[60] While the outcome of patients with *IKZF1*-altered, Ph-like ALL was dismal, there was no significant difference among Ph-like patients with respect

to *IKZF1* status. However, the frequency of *IKZF1*-alterations in this group was very high (86%). Among our patients with *PAX5*-altered genotypes, PAX5 P80R had a favorable outcome, similar[61] or better than reported,[27,34] and significantly better than PAX5alt patients.[27] While *ZNF384*-R/-like patients had an intermediate molecular risk, as reported,[33,34,37,38,62] so did *MEF2D-R* patients, who did better than previously suggested.[33,34,40,41] Our large group of adult *DUX4*-R patients confirmed the excellent outcome seen in children and young adults.[27,30-32]

Genomic analysis revealed that, despite a median age of 35 years, one-third of our patients had a favorable molecular risk. Importantly, E2993 patients deemed high-risk based on age and presenting WBC count who were molecularly standard- or intermediate-risk did significantly better than molecular high-risk patients within the protocol-defined high-risk cohort. However, younger age and lower WBC count still positively affected OS both in the standard- and high-risk molecular subgroups (Figure 4), highlighting the importance of combining genomic analysis with traditional prognostic factors for risk stratification.

In summary, our results have important clinical implications. We have demonstrated that molecular subtypes differ strikingly in their responses to treatment on a single trial. While MRD is another accepted powerful prognostic indicator in adult B-ALL,[63,64] its impact in genetic subgroups has rarely been analyzed. Ph-like ALL patients tend to remain MRD-positive after induction chemotherapy.[16,17,20,65] In children, MRD was prognostically important for Ph-like or hypodiploid ALL.[65,66] Other trials of Ph-like ALL,[16,67] however, suggested that relapse-risk remained high despite risk-adapted therapy and outcomes remained

poor even if MRD-negativity was achieved. Response kinetics, clinically relevant cut-off levels and optimal timing of MRD testing vary between low- and high-risk genotypes.[68,69] When UKALLXII/E2993 was activated in 1993, MRD testing was not yet standard of care in adult B-ALL. As a result, submission of follow-up specimens was not mandated, limiting our potential for MRD testing. The MRC assessed MRD in 161/820 UKALLXII *BCR/ABL1*-negative B-ALL patients (19.6% of their accrual) and demonstrated that MRD was a predictor of relapse both in protocol-defined standard- and high-risk patients, though cohorts were small.[70] While we could not answer the interaction of MRD and molecular genotype in the present study, we will get clarity from the recently completed phase 3 trial E1910 (ClinicalTrials.gov NCT02003222), in which flow cytometric MRD was determined throughout the course of disease, samples underwent multimodal genetic analyses, and treatment with blinatumomab was assigned or randomized based on MRD status.

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Authorship

Contribution: E.P., K.G.R., C.G.M. and M.R.L. prepared the manuscript; E.P., K.G.R., Z.G., J.B., J.R., Y.Z., R.L.L., O.A.-W., Z.C; G.W.; C.Q.; L.S.; S.P.; C.L.W., R.H., L.F., B.P., A.M. and D.A. performed experiments and data analyses and helped with data interpretation; V.W., G.A.N.B., D.P. and C.C. performed statistical analyses; Y.Z. G.W.D., and A.V.M. centrally reviewed institutional cytogenetic data;

H.M.L., S.M.L., A.K.F., D.I.M., P.H.W., J.M.R., M.S.T. and A.H.G. organized the clinical trial; all authors reviewed and approved the manuscript.

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Table 1: Baseline characteristics of 251 molecularly characterized E2993 patients (excluding the 31 B-other patients), including patients who were not eligible for trial

Molecular subgroup	**Number of patients**	**%**	**Age (years) median (Q1,Q3)**	**WBC x 10^9/L median (Q1,Q3)**	**Sex (%) F/M**
DUX4-R	22	7.8	26.0 (22.5,40.3)	9.3 (3.5,23.6)	52.4/47.6
ETV6-RUNX1/-like*	5	1.8	22.0 (20.0,26.0)	6.7 (2.0,9.7)	60.0/40.0
TCF3-PBX1	15	5.3	29.0 (22.5,37.5)	29.8 (10.4,61.2)	46.7/53.3
KMT2A-AFF1	27	9.6	38.0 (32.0,45.0)	92.0 (43.4,229.2)	76.9/23.1
KMT2A-non-*AFF1*	10	3.5	35.5 (31.5,46.5)	84.4 (6.1,190.2)	70/30
PAX5alt	27	9.6	38.0 (20.5,50.0)	9.2 (3.5,23.5)	18.5/81.5
PAX5 P80R	10	3.5	26.5 (20.8,46.3)	11.7 (3.9,22.5)	30/70
Ph-like *CRLF2*-R	30	10.6	28.0 (19.3,35.8)	23.3 (11.0,70.8)	20/80
Ph-like non-*CRLF2*-R	18	6.4	38.5 (30.0,48.8)	14.3 (6.1,60.4)	61.1/38.9
ZNF384-R/-like*	11	3.9	27.0 (26.0,42.5)	13.2 (6.2,36.7)	18.2/81.8
MEF2D-R	6	2.1	22.0 (19.0,43.8)	25.0 (10.0,32.9)	50.0/50.0
BCL2/MYC	12	4.3	40.0 (26.5,48.8)	28.5 (16.7,57.8)	36.4/63.6
ZEB2/CEBPE	4	1.4	43.0 (37.8,45.8)	32.6 (2.8,86.9)	50.0/50.0
iAMP21	3	1.1	21.0 (20.0,30.0)	4.2 (3.9,6.0)	33.3/66.7
TCF3-HLF	1	0.4	44 (44,44)	6.1 (6.1,6.1)	100/0
High Hyperdiploid	16	5.7	20.0 (18.0,29.3)	2.5 (1.9,9.5)	25/75
Low Hypodiploid/ Near Haploid	34	12.1	44.0 (36.0,53.0)	3.7 (2.9,7.6)	37.1/62.9

*, indicates combination of *ETV6-RUNX1* and *ETV6-RUNX1*-like as well as *ZNF384* and *ZNF384*-like patients

Table 2: Gene rearrangements and gene mutations of 251 molecularly characterized E2993 patients (excluding 31 B-other patients)

Molecular Subgroup	Gene rearrangements and mutations (# of patients)
DUX4-R	IGH-DUX4 (19), MZF1-DUX4 (1), DUX4 overexpression (2)
ETV6-RUNX1	ETV6-RUNX1 (3)
ETV6-RUNX1-like	IKZF1-FIGNL1 (1), no fusion (1)
TCF3-PBX1	TCF3-PBX1 (15)
TCF3-HLF	TCF3-HLF (1)
KMT2A-R	KMT2A-AFF1 (27), KMT2A-MLLT1 (5), KMT2A-EPS15 (2), KMT2A-MLLT3 (1), KMT2A-AFF1^{NEG} by PCR (2)
Ph-like *CRLF2*-R	IGH-CRLF2 (19), P2RY8-CRLF2 (6), IGH-CRLF2 + P2RY8-CRLF2 (1), USP9X-RBM10 (1), high CRLF2 expression and Ph-like score (2), NA (1)
Ph-like non-*CRLF2*-R	PAX5-JAK2 (2), SSBP2-JAK2 (2), PCM1-JAK2 (1), ZNF340-JAK2 (1), ZNF340-TYK2 (1), RCSD1-ABL1 (1), RCSD1-ABL2 (1), ETV6-NTRK3 (1), IGH-EPOR (1), IGK-EPOR (1), FIP1L1-PDGFRA (1), IL7R p.L242ins (1), no fusion (4)
PAX5alt	PAX5-ETV6 (4), PAX5-ZNF318 (1), IGH-CRLF2 (1), P2RY8-CRLF2 (1), PAX5 G334fs (1), PAX5 R140L (1), PAX5 R59W (1), no fusion or mutation (17)
PAX5 P80R	P2RY8-CRLF2 (1), PAX5 p.Pro80Arg (9)
ZNF384-R	EP300-ZNF384 (6), TCF3-ZNF384 (2), TAF15-ZNF384 (1)
ZNF384-like	ZBT47-ZNF652 (1), no fusion (1)
MEF2D-R	MEF2D-BCL9 (5), MEF2D-SS18 (1)
BCL2/MYC	IGH-MYC (3), IGH-BCL2 (2), IGH-BCL2 + BACH2-MYC (1), REPS1-BCL2 (1), IGH-BCL2 + IGH-MYC (2), SMC4-BCL6 (1), IGH-BCL2 + MBNL1-BCL6 (1), IGH-BCL6 (1),
ZEB2/CEBPE	IGH-CEBPE (3), no fusion (1)
iAMP21	IGH-CRLF2 (1), no fusion (2)
HH	no fusion (9), NA (7)
LH/NH	CREBBP-UTRN (1), TFAP4-TRAP1 (1), PAX5 R140L (1), no fusion (29), NA (3)

no fusion, inability to detect a fusion with the methodology used; NA, RNA-seq was not performed

Table 3: Univariable Cox-model of relapse-free (RFS) and overall survival (OS) for molecular subgroups compared to the *DUX4*-R patients as reference group

Molecular subgroup	**RFS Hazard Ratio**	**P-value**	**OS Hazard Ratio**	**P-value**
BCL2/MYC	36.4 (7.9, 167.2)	<0.001	11.3 (3.2, 39.3)	<0.001
KMT2A-AFF1	7.8 (2.6, 23.3)	<0.001	6.6 (2.5, 17.1)	<0.001
LH/NH	7.6 (2.6, 22.4)	<0.001	5.3 (2.0, 14.1)	<0.001
Ph-like	7.5 (2.7, 21.3)	<0.001	4.9 (1.9, 12.6)	<0.001
MEF2D-R	5.9 (1.6, 21.8)	0.009	3.0 (0.8, 11.3)	0.098
PAX5alt	4.6 (1.5, 13.7)	0.006	3.7 (1.4, 10.0)	0.010
ZNF384-R/-like	4.0 (1.1, 14.3)	0.031	2.6 (0.8, 8.6)	0.111
HH	2.4 (0.7, 8.4)	0.154	1.0 (0.3, 3.7)	0.995
ETV6-RUNX1/-like	2.0 (0.4, 11.1)	0.411	0.8 (0.1, 6.7)	0.819
PAX5 *P80R*	1.5 (0.3, 6.8)	0.585	1.2 (0.3, 5.1)	0.795
TCF3-PBX1	1.2 (0.3, 5.5)	0.781	1.6 (0.5, 5.7)	0.429

Table 4: 5-year relapse-free (RFS) and overall survival (OS) of E2993 patients by combined protocol-defined and molecular-defined risk assignment.

Combined risk group	**5-year RFS %** (95% confidence interval)	**5-year OS %** (95% confidence interval)
pLR/mSR	73.3 (59.8, 89.9)	87.9 (77.5, 99.8)
pLR/mIR	29.4 (14.1, 61.4)	31.6 (16.3, 61.2)
pLR/mHR	28.9 (14.7, 56.6)	36.2 (21.5, 60.9)
pHR/mSR	62.0 (45.2, 85.1)	53.2 (37.5, 75.6)
pHR/mIR	27.7 (13.8, 55.3)	42.0 (25.5, 69.4)
pHR/mHR	11.9 (6.23, 22.9)	12.9 (7.19, 23.1)

pLR, protocol-defined low-risk; pHR, protocol-defined high-risk; mSR, molecular standard-risk; mIR, molecular intermediate-risk; mHR, molecular high-risk;

FIGURE LEGENDS

Figure 1. Antigen expression in major molecular subgroups. Unsupervised hierarchical clustering of antigen expression in each of 413 E2993 patients. The patient cohort consists of 244 samples from *BCR-ABL1* negative E2993 patients which were genotyped (excluding molecular subgroups with <5 patients and B-other) and 169 BCR-ABL1 E2993 ALL patients. This approach identified characteristic antigen expression profiles within each of the 13 molecular subtypes, shown as black bars above the heatmap. Columns represent patients and rows are antigens. Antigens are listed to the right of the heatmap. Expression levels reflect the percentage of antibody-binding leukemic lymphoblasts, except for CD38_mfi, which reflects intensity of antigen expression. High- and low-expression of antigens in the heatmap are shown in shades of red and blue, respectively. The first row above the heatmap aligns genotypes with B-lineage maturation stages. ZNF384-R includes ZNF384-like; ETV6-RUNX1 includes ETV6-RUNX1-like; KMT2A-R includes both KMT2A-AFF1 and KMT2A-non-AFF1; low_hypo, low-hypodiploid and near-haploid patients; mcd22, membrane (surface) CD22;

Figure 2. Overall survival of BCR/ABL1-negative B-ALL patients on UKALLXII/E2993 by matched sibling donor availability at 10 years.

Figure 3. Outcome of E2993 Molecular Subgroups. Kaplan-Meier estimates for relapse-free (RFS) and overall survival (OS) of molecular subgroups included in the outcome analysis. The RFS analysis included only patients who achieved a

complete remission (*DUX4*-R, 18 patients; *TCF3-PBX1*, 12; PAX5 P80R, 9; Hyperdiploid, 15; *ETV6-RUNX1*/-like, 5; *ZNF384*-R/-like, 10; PAX5alt, 24; *MEF2D*-R, 6; *KMT2A-AFF1*, 21; Ph-like, 41; Hypodiploid/Haploid, 24; *BCL2/MYC*, 3). The OS analysis included all patients with outcome information (*DUX4*-R, 20 patients; *TCF3-PBX1*, 15; PAX5 P80R, 9; Hyperdiploid, 15; *ETV6-RUNX1*/-like, 5; *ZNF384*-R/-like, 11; PAX5alt, 27; *MEF2D*-R, 6; *KMT2A-AFF1*, 26; Ph-like, 46; Hypodiploid/Haploid, 31; *BCL2/MYC*, 6). Among *DUX4*-R, *ETV6-RUNX1*/-like, *TCF3-PBX1*, PAX5 P80R and hyperdiploid patients, neither RFS nor OS varied significantly.

Figure 4. Outcome of E2993 patients by protocol-defined and molecular-defined risk assignment. Kaplan-Meier estimates of relapse-free (RFS) and overall survival (OS) for patients stratified by their protocol-defined risk: low-risk (pLR) and high-risk (pHR), and molecular risk: standard-risk (mSR), intermediate-risk (mIR), and high-risk (mHR).

Figure 1: Antigen expression in major molecular subgroups.

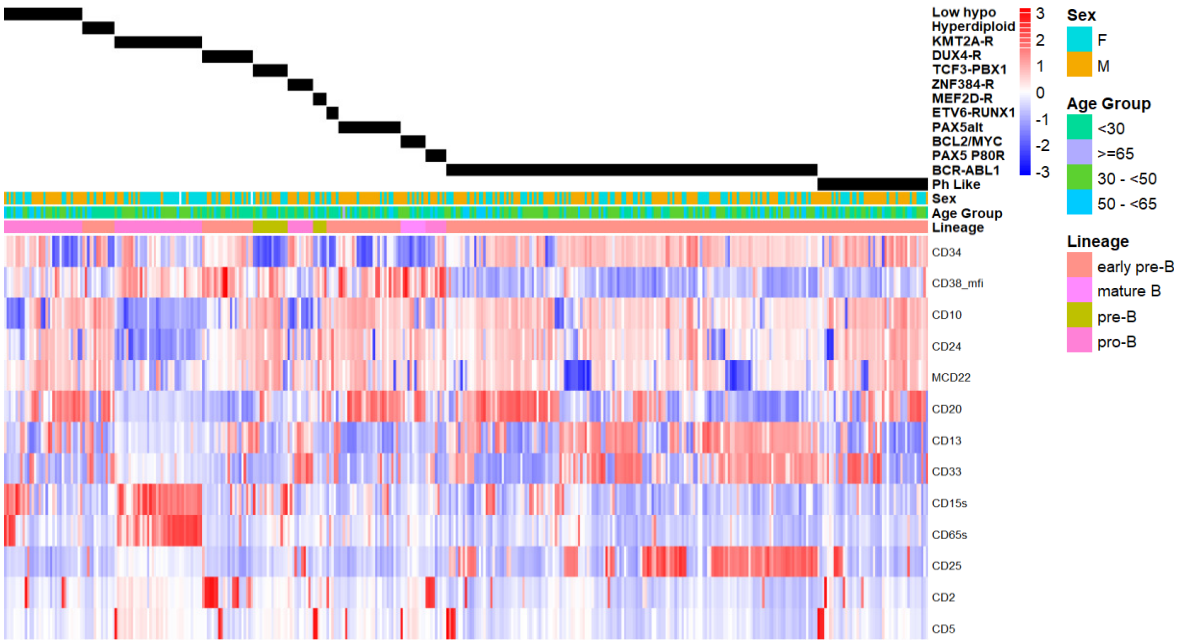

Figure 2 Overall survival of *BCR/ABL1*-negative B-ALL patients on UKALLXII/E2993 by matched sibling donor availability at 10 years.

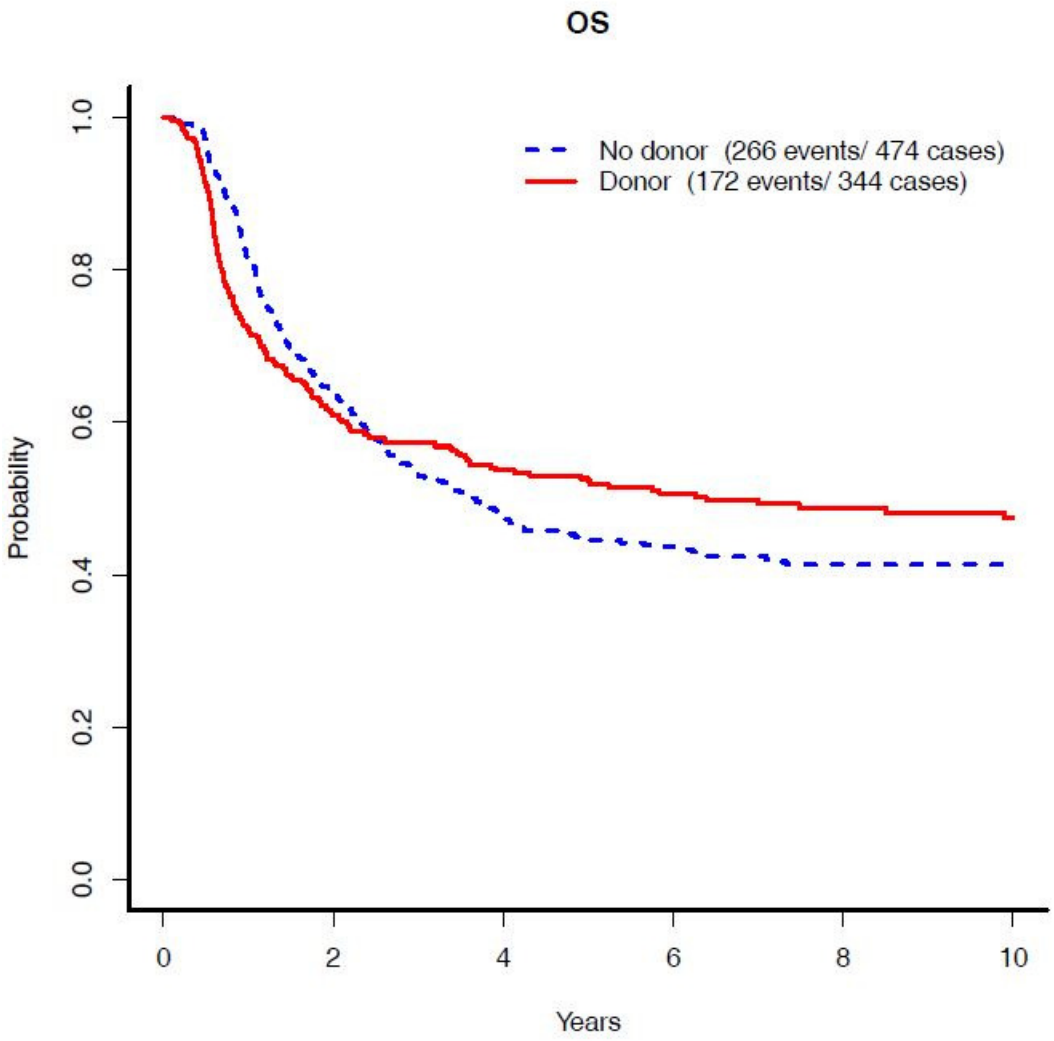

Figure 3 Outcome of E2993 Molecular Subgroups.

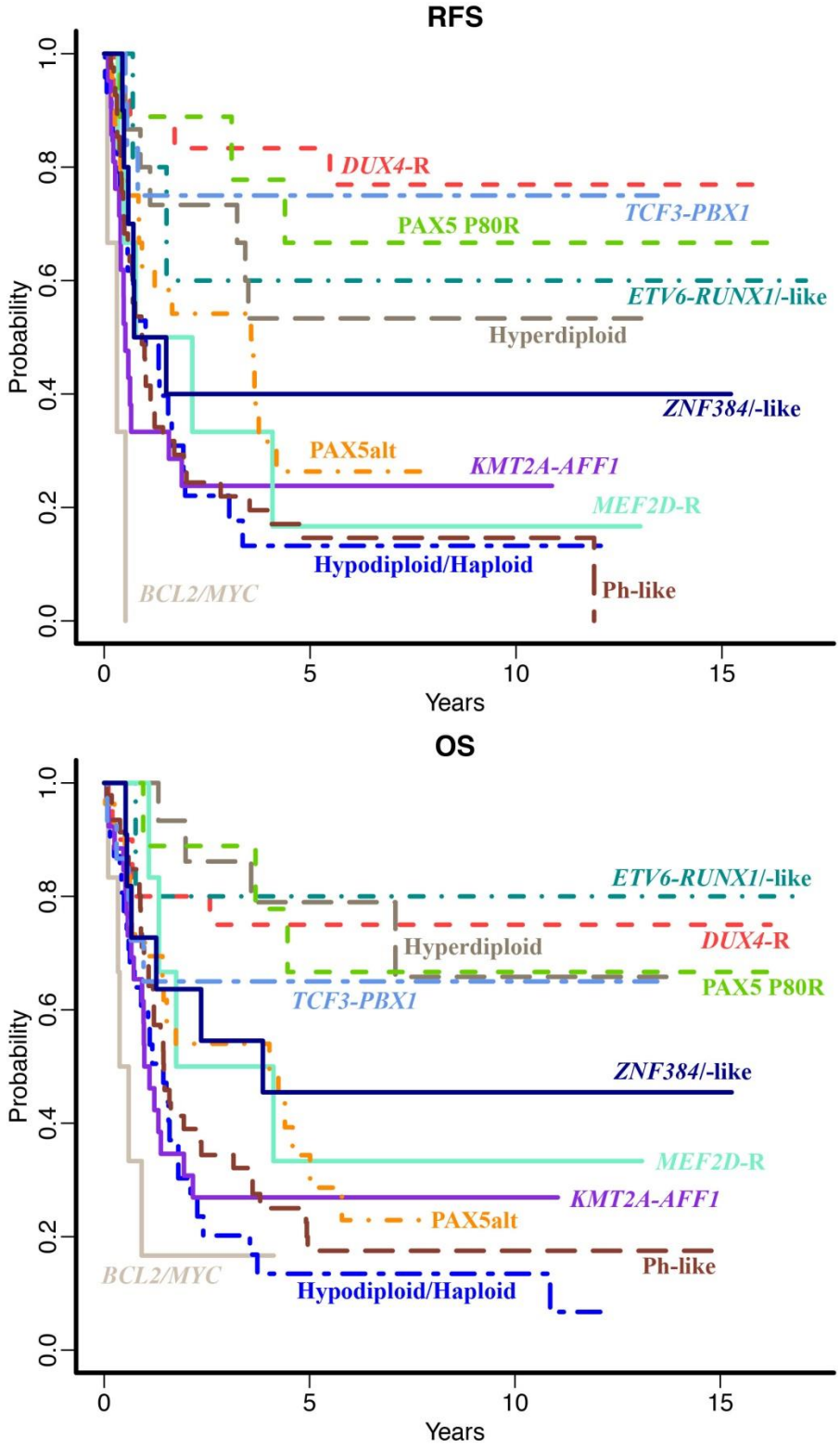

Figure 4: Outcome of E2993 patients by protocol-defined and molecular risk assignment.