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Prognostic implications of additional genomic lesions in adult Philadelphia chromosome-positive acute lymphoblastic leukemia

Anna Lucia Fedullo,[1]* Monica Messina,[1]* Loredana Elia,[1] Alfonso Piciocchi,[2] Valentina Gianfelici,[1] Alessia Lauretti,[1] Stefano Soddu,[2] Maria Cristina Puzzolo,[1] Clara Minotti,[1] Felicetto Ferrara,[3] Bruno Martino,[4] Patrizia Chiusolo,[5] Valeria Calafiore,[6] Stefania Paolini,[7] Marco Vignetti,[1,2] Antonella Vitale,[1] Anna Guarini,[8] Robin Foà[1]* and Sabina Chiaretti[1]*

[1]Hematology, Department of Translational and Precision Medicine, Sapienza University, Rome; [2]GIMEMA Data Center, Rome; [3]Division of Hematology and Stem Cell Transplantation Unit, Cardarelli Hospital, Naples; [4]Hematology Unit, Azienda Ospedaliera Bianchi Melacrino Morelli, Reggio Calabria; [5]Institute of Hematology, Catholic University, Rome; [6]Division of Hematology, AOU Policlinico, University of Catania; [7]"L. and A. Seràgnoli" Institute of Hematology, University of Bologna and [8]Department of Molecular Medicine, Sapienza University, Rome, Italy

These authors contributed equally to this work

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Correspondence:

chiaretti@bce.uniroma1.it or
rfoa@bce.uniroma1.it

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ABSTRACT

To shed light onto the molecular basis of Philadelphia chromosome-positive acute lymphoblastic leukemia and to investigate the prognostic role of additional genomic lesions, we analyzed copy number aberrations using the Cytoscan HD Array in 116 newly diagnosed adult patients with Philadelphia chromosome-positive acute lymphoblastic leukemia enrolled in four different GIMEMA protocols, all based on a chemotherapy-free induction strategy. This analysis showed that patients with Philadelphia chromosome-positive acute lymphoblastic leukemia carry an average of 7.8 lesions/case, with deletions outnumbering gains (88% *versus* 12%). The most common deletions were those targeting *IKZF1*, *PAX5* and *CDKN2A/B*, which were detected in 84%, 36% and 32% of cases, respectively. Patients carrying simultaneous deletions of *IKZF1* plus *CDKN2A/B* and/or *PAX5* had a significantly lower disease-free survival rate (24.9% *versus* 43.3%; *P*=0.026). The only *IKZF1* isoform affecting prognosis was the dominant negative one (*P*=0.003). Analysis of copy number aberrations showed that 18% of patients harbored *MEF2C* deletions, which were of two types, differing in size: the longer deletions were associated with the achievement of a complete molecular remission (*P*=0.05) and had a favorable impact on disease-free survival (64.3% *versus* 32.1% at 36 months; *P*=0.031). These findings retained statistical significance also in multivariate analysis (*P*=0.057). *KRAS* deletions, detected in 6% of cases, were associated with the achievement of a complete molecular remission (*P*=0.009). These results indicate that in adults with Philadelphia chromosome-positive acute lymphoblastic leukemia a detailed evaluation of additional deletions - including *CDKN2A/B*, *PAX5*, *IKZF1*, *MEF2C* and *KRAS* - has prognostic implications and should be incorporated in the design of more personalized treatment strategies.

Introduction

The Philadelphia (Ph) chromosome derives from the t(9;22)(q34;q11) and leads to a *BCR-ABL1* rearrangement.[1] The incidence of this chromosomal change in acute lymphoblastic leukemia (ALL) increases with age, being detected in 25% of adults and in about 50% of elderly patients.[2] Prior to the advent of tyrosine kinase inhibitors, the outcome of Ph+ ALL patients was extremely poor,[3-5] and the only

possibility of a cure was allogeneic stem cell transplantation (HSCT), when feasible.[6,7] The introduction of tyrosine kinase inhibitors, administered with low doses or without chemotherapy during induction, followed by consolidation chemotherapy and HSCT has markedly improved the management and outcome of adult Ph+ ALL patients, with survival rates at 5 years now approaching 50%.[8-17]

Different biological features - the type of fusion transcript (i.e. p190 or p210),[18] the persistence and/or reappearance of minimal residual disease (MRD),[19,20] additional genomic deletions (particularly *IKZF1*, and to a lesser extent *CDKN2A/B* and *PAX5*[21-24]) - and the presence of mutations at relapse are associated with a worse outcome.[25-27] However, a broad and refined biological algorithm that could help to optimize treatment strategies and define better whether some patients could be spared intensive treatment, including HSCT, has so far not been proposed.

To this end, in the present study we investigated copy number aberrations (CNA) in 116 newly diagnosed adult Ph+ ALL patients to identify additional molecular lesions with the aim of improving patients' stratification and management.

Methods

Experimental strategy

Bone marrow and/or peripheral blood samples from 116 patients (Table 1) with newly diagnosed Ph+ ALL enrolled in four GIMEMA (*Gruppo Italiano Malattie EMatologiche dell'Adulto*) trials were analyzed (*Online Supplementary Table S1*). The study was carried out in four phases (*Online Supplementary Figure S1*): (i) CNA analysis of 116 samples by Cytoscan; (ii) multiplex ligation-dependent probe amplification analysis; (iii) validation of *MEF2C* deletions by digital droplet (dd) polymerase chain reaction (PCR); and (iv) *MEF2C* and *KRAS* mutational screening.

This study was approved in the context of an *Associazione Italiana per la Ricerca sul Cancro* (AIRC) project (10007) with Institutional Review Board number 2182/16.06.2011.

Copy number aberration analysis

CNA were analyzed using CytoScan® HD Arrays (Affymetrix, Santa Clara, CA, USA) and Chromosome Analysis Suite (ChAS) software. Germline material from five paired samples was also evaluated. Recurrent deletions were validated with the Salsa MLPA P335 ALL-IKZF1 kit (MRC-Holland, Amsterdam, the Netherlands)[28,29] (*Online Supplementary Data*). Statistical analyses on clinical correlates are described in the *Online Supplementary Data*.

Table 1. Patients' clinical features.

	Patients (n=116)
Gender (male/female)	55/61
Age, years (range)	51.1 (18.9-89)
Median white cell count x 10^9/L (range)	25.4 (1.7-597)
Median hemoglobin g/dL (range)	9.6 (4-16.3)
Median platelet count x 10^9/L (range)	50 (4-333)
Fusion transcript (p190/p210/p190-210)¥	70/29/16
Complete molecular response* yes/no	17/99

*As per protocol definition; ¥information missing for one patient.

Digital droplet polymerase chain reaction assays

MEF2C deletions were validated by ddPCR using the QX200™ Droplet Digital™ PCR System (BioRad, Hercules, CA, USA) and QuantaSoft Analysis Pro software according to the manufacturer's instructions (*Online Supplementary Data*).

Mutational screening

Sanger sequencing of PCR products for *MEF2C* and *KRAS* exons (*Online Supplementary Table S2*) was performed with the ABI-Prism 3500 sequencer (Applied Biosystem, Life Technologies, Foster City, CA, USA) (*Online Supplementary Data*).

Results

Copy number aberration analysis

CNA analysis revealed 7.8 aberrations/patient (range: 0-28), the majority being losses (88%) with only 12% gains, both spanning from whole chromosomes to focal lesions;[22,23,30-32] no differences were recorded among trials (Figure 1A).

Gross chromosomal lesions were found in 42% of cases: the majority were losses of chromosome 7 (18.1%), followed by monosomy of chromosome 9 or 9p deletion (9%) and gain of 1q (7.7%) (Figure 1B, *Online Supplementary Table S3*). Smaller deletions - limited to one to three genes and defined as focal - were found in 56% of cases.

The most frequently deleted region involved the 7p12 cytoband comprising *IKZF1*[22,23,33,34] which was detected in 97 cases (84%).

PAX5 was deleted in 43 patients (36.2%), while 37 (31.9%) had deletions of *CDKN2A/B*. *MLLT3*, *BTG1*, *BTLA*, *CD200* and *RB1* were deleted in 30, 27, 21, 17 and 16 cases, respectively (25.9%, 23%, 18.1%, 17.2%, 14.6%, and 13.8%) (Figure 1C).

IKZF1 deletions (*ΔIKZF1*) occurred together with *CDKN2A/B* and/or *PAX5* deletions in 45/97 cases (46.4%) and are defined as *ΔIKZF1+CDKN2A* and/or *PAX5* (Figure 1D): this subset displayed similar lesions to those recently described by Stanulla and colleagues.[35] With regard to potential interactions, we found a significant association between *IKZF1* and *PAX5* deletions (P=0.01), but not with *CDKN2A*.

Multiplex ligation-dependent probe amplification confirmed *IKZF1*, *PAX5*, *CDKN2A*, *BTG1*, *EBF1*, *ETV6* and *RB1* lesions, and allowed evaluation of *IKZF1* isoforms. These isoforms were grouped into four classes:[24,36] wild-type, dominant-negative (comprising Δ4-7 cases, 29.8%), haploinsufficient (including all cases harboring a deletion that involves exon 2 - i.e. Δ2-7, Δ2-8, Δ2-3, Δ1-3 - or the whole gene, 57.7%) and miscellaneous (remaining cases, 11.3%).

Identification of novel lesions

CNA analysis highlighted additional genomic lesions (Table 2, *Online Supplementary Table S4*). We focused in particular on *MEF2C* and *KRAS* deletions since these had prognostic significance (see below). *MEF2C* deletions were detected in 21 patients (18.1%) and differed in size. According to the length of intron 1-2 losses, deletions were grouped into two categories. One category - detected in 14 cases (67% of *MEF2C* deleted cases) - was characterized by a larger minimal common region (6.2 Kb) involving introns 1-2 and exon 2 (the first codifying exon),

defined Δ*MEF2C*-long. The other category, detected in seven patients, was smaller (5.4 Kb) and involved only exon 2, and was called Δ*MEF2C*-short (Figure 2A). ddPCR confirmed *MEF2C* lesions in all cases. No *MEF2C* mutations were identified.

KRAS deletions (Δ*KRAS*) were detected in seven cases (6%); the focal lesion of *KRAS* started in the 5' untranslated region and ended in intron 1-2, involving the first non-codifying exon (Figure 2B). The minimal common region consisted of 135 Kb. *KRAS* was not affected by mutations.

Impact of known and novel deletions on complete molecular response achievement and disease-free survival

We did not find significant differences between patients with Δ*IKZF1* and *IKZF1* wild-type cases with regard to achievement of complete molecular response (CMR) or disease-free survival (DFS) (*Online Supplementary Figure S2*). Further stratification according to *IKZF1* isoforms showed that patients with the dominant-negative isoform had a lower DFS rate (23.3%; *P*=0.039) compared to patients with the other isorforms, particularly wild-type (53.3%; *P*=0.016) and haploinsufficient cases (40.3%; *P*=0.015); the DFS rate of the miscellaneous group (34.1%) did not differ significantly from that of the dominant-negative cases (Figure 3A). These differences were not statistically significant in the overall survival analysis (Figure 3B).

We also investigated the outcome of Δ*IKZF1*+*CDKN2A* and/or *PAX5* cases. The CMR rate did not differ between Δ*IKZF1*+*CDKN2A* and/or *PAX5* and Δ*IKZF1*-only cases; contrariwise, Δ*IKZF1*+*CDKN2A* and/or *PAX5* patients had a significantly worse DFS than Δ*IKZF1*-only cases (43.3% *versus* 24.9%; *P*=0.026) (Figure 3C) and an inferior overall survival (62.6% *versus* 40.2%; *P*=0.02) (Figure 3D).

The presence of Δ*MEF2C*-long was associated with a higher rate of CMR achievement (*P*=0.05); this effect was not influenced by the protocol or the tyrosine kinase inhibitor used (imatinib or dasatinib). Furthermore, Δ*MEF2C*-long cases were also associated with a signifi-

Table 2. Minimal common region (MCR) of identified focal lesions.

Deleted gene	N. of patients (%)	MCR (hg19)
FOCAD	29 (25)	chr9: 20685149 - 20759956
CDK6	24 (20.7)	chr7: 92456635 - 92266647
PTPRD	21 (18.1)	chr9: 8153932 - 8854489
MEF2C	21 (18.1)	chr5:88122179 - 88127630
BTLA	21 (18.1)	chr3:112154702 - 112217769
JAK2	20 (17.2)	chr9: 5123013 - 5234403
ADD3	18 (15.5)	chr10: 111795029 - 111853667
SLX4IP	17 (14.6)	chr20: 10417444 - 10451891
CD200	17 (14.6)	chr3:112054292 - 112217769
HBS1L	16 (13.7)	chr6: 135375338 - 135418257
ATP10A	14 (12)	chr15: 26055568 - 26103185
TOX	8 (6.9)	chr8:60028851 - 60110235
KRAS	7 (6)	chr12: 25402194 - 25537468
ARHGAP24	7 (6)	chr4:86493655 - 86436188
EBF1	6 (5.1)	chr5: 158440156 - 158164599
LEF1	5 (4.3)	chr4:109034392 - 109084557
MDM2	5 (4.3)	chr12:69159076 - 69205287
TCF12	4 (3.4)	chr15:57294905 - 57399047
ERG	2 (1.7)	chr21:39772775 - 39788683

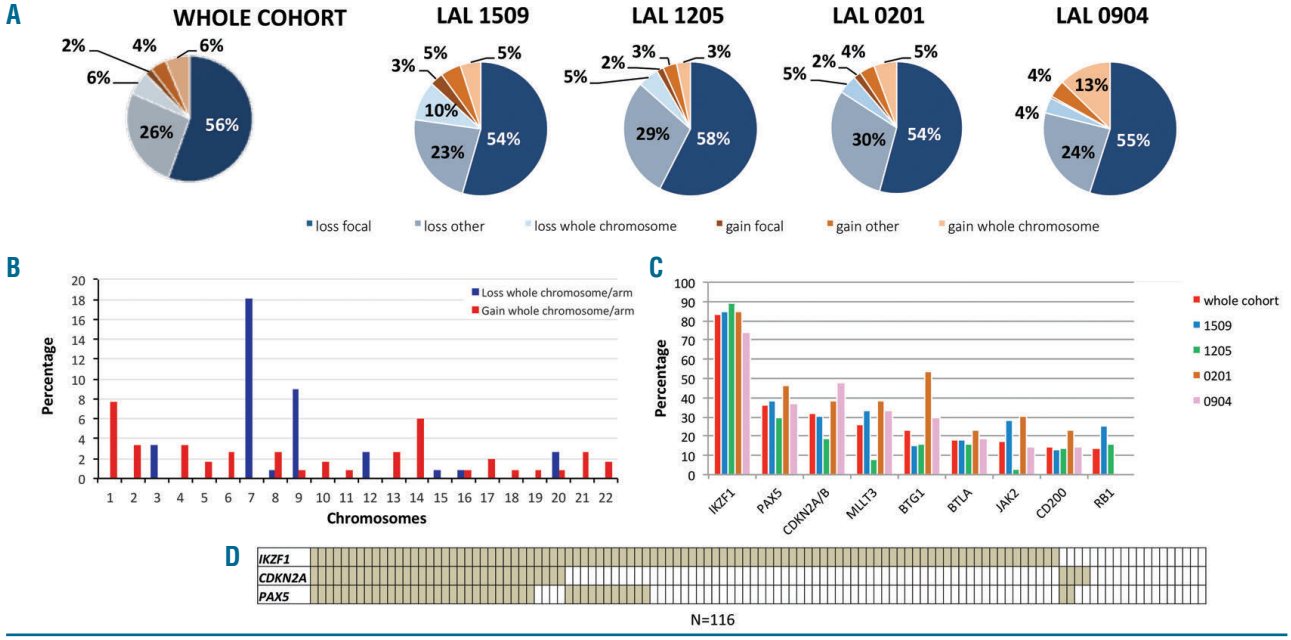

Figure 1. Overall load and incidence of genetic lesions in Philadelphia chromosome-positive acute lymphoblastic leukemia. (A) Distribution of copy number aberrations in the whole cohort and across different protocols. (B) Percentages of gross chromosomal aberrations. (C) Percentages of deletions of known genes in the whole cohort (n=116) and in the different studies analyzed. (D) Heatmap of *IKZF1*, *CDKN2A/B*, and *PAX5* deletions in the whole cohort.

cantly better DFS (64.3% *versus* 32.1%; *P*=0.031) (Figure 4A) and overall survival (77.9% *versus* 48.4%; *P*=0.036) (Figure 4B).

Lastly, Δ*KRAS* was more frequently found in patients who obtained a CMR (24% *versus* 3%; *P*=0.009), but this finding did not have an impact on DFS.

Prognostic impact of known and novel genomic lesions in univariate and multivariate analyses

In univariate analysis, Δ*MEF2C*-long and Δ*KRAS* had an impact on CMR achievement, while Δ*MEF2C*-long and Δ*IKZF1+CDKN2A* and/or *PAX5* influenced DFS (Table 3).

In multivariate analysis for CMR, performed taking into

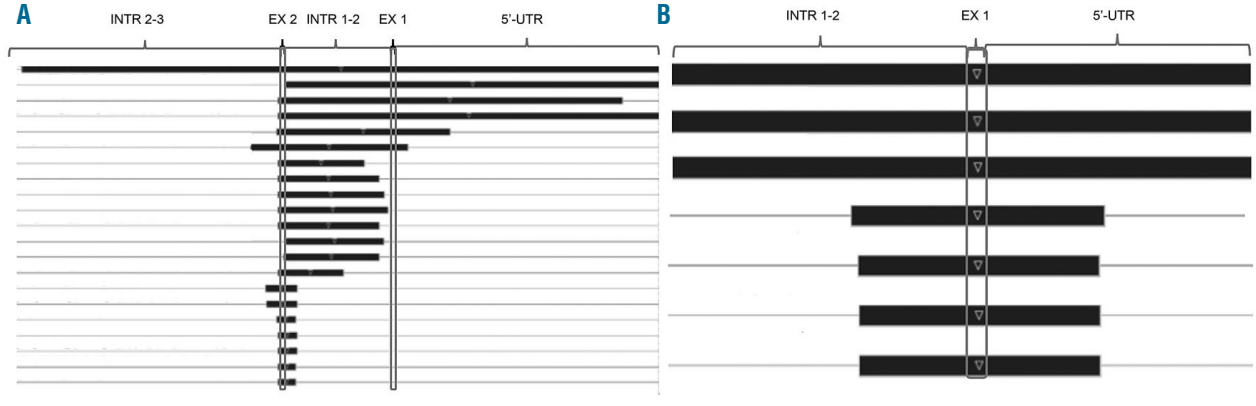

Figure 2. Representation of Δ*MEF2C* and Δ*KRAS*. (A) Representation of Δ*MEF2C* for each patient. Lesions are ordered according to their size: one case had a deletion of the whole gene, one had a deletion that involved only exon 1 spanning from intron 1-2 to the 5' untranslated region (5'UTR), four had deletions starting from intron 2-3 and ending at 5'-UTR, thereby involving both exons 2 and 1 (the latter being an untranslated exon), 13 had lesions spanning from intron 2-3 to intron 1-2, therefore involving exon 2 (the first codifying exon), with six of them harboring a longer intron 1-2 deletion. Lastly, two cases had deletions that involved only intron 1-2. The first 14 cases were considered as Δ*MEF2C*-long and the remaining as Δ*MEF2C*-short. (B) Representation of Δ*KRAS* for each patient. Lesions are ordered according to their size: in four cases, the deletion encompassed only *KRAS* itself, whereas in three it involved the short arm of chromosome 12. INTR: intron; EX: exon; 5'UTR: 5' untranslated region.

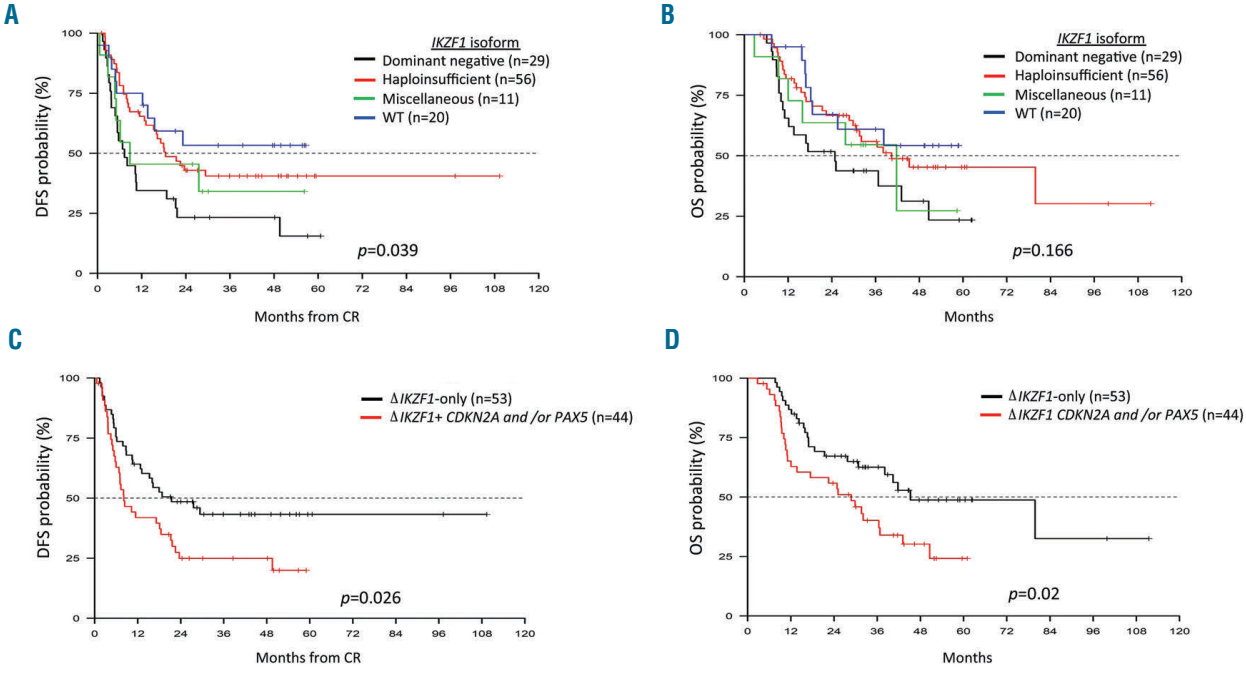

Figure 3. Survival probability curves according to *IKZF1* status. (A) Disease-free survival and (B) overall survival at 36 months of patients divided according to *IKZF1* isoform. (C) Disease-free survival and (D) overall survival at 36 months of Δ*IKZF1*-only and Δ*IKZF1+ CDKN2A* and/or *PAX5* patients. WT: wild-type; DFS: disease-free survival; OS: overall survival; CR: complete remission.

account white blood cell count, age, tyrosine kinase inhibitor use and the genomic lesions described above, the only factor that retained statistical significance was Δ*KRAS* (*P*=0.01); a trend was noted for Δ*MEF2C*-long deletions (*P*=0.075) (Table 3).

In multivariate analysis for DFS, considering Δ*MEF2C*-long, Δ*IKZF1+CDKN2A* and/or *PAX5*, white blood cell count and CMR as variables, the factors that had a negative impact were Δ*MEF2C*-long (*P*=0.057) and white blood cell count (*P*=0.05), while a trend towards a worse DFS was observed for Δ*IKZF1+CDKN2A* and/or *PAX5* (*P*=0.089) (Table 3). HSCT did not affect the prognostic role of the above-mentioned lesions.

Discussion

The management of adults with Ph+ ALL currently relies on the use of first,[8-10,13-16] second[11,12] and third[37] generation tyrosine kinase inhibitors, either alone[9-12] or in combination with chemotherapy,[8,13-16,37] followed - if feasible and necessary - by HSCT. These approaches have greatly improved the outcome of Ph+ ALL: nowadays, virtually all patients - independent of age - achieve a complete hematologic remission, coupled to a CMR in a variable proportion of cases. Nonetheless, in all reported studies the long-term outcome is in the range of 50% at 5 years; thus, additional prognosticators capable of better stratify-

Table 3. Summary of univariate and multivariate analyses for complete molecular response and disease-free survival for the factors identified.

	Univariate analysis for CMR OR (95% CI)	*P* value	Multivariate analysis for CMR OR (95%CI)	*P* value
MEF2C deletions	0.288 (0.082, 1007)	0.051	0.259 (0.058, 1.146)	0.075
KRAS deletions	0.12 (0.024, 0.601)	0.009	0.068 (0.009, 0.529)	0.01
White blood cell count	0.986 (0.969, 1.003)	0.1	0.986 (0.966, 1.007)	0.188
Age	1.026 (0.99, 1.063)	0.16	1.028 (0.985, 1.072)	0.205
Imatinib *vs.* dasatinib	0.267 (0.057, 1.248)	0.093	0.296 (0.054, 1.615)	0.159
Fusion protein (p190 *vs.* p210 and p190/210)	1.247 (0.421, 3.693)	0.691		
Δ*IKZF1+ CDKN2A* and/or *PAX5*	1.600 (0.599, 4.581)	0.381		
	Univariate analysis for DFS HR (95% CI)	***P* value**	**Multivariate analysis for DFS HR (95%CI)**	***P* value**
MEF2C deletions	0.359 (0.144, 0.891)	0.027	0.417 (0.169-1.028)	0.057
Δ*IKZF1+ CDKN2A* and/or *PAX5*	1.834 (1.148, 2.929)	0.011	1.608 (0.930, 2.781)	0.089
White blood cell count	1.002 (1, 1.004)	0.065	1.003 (1, 1.006)	0.050
Age	1.001 (0.986, 1.017)	0.866	1.01 (0.995-1.028)	0.180
CMR	0.423 (0.181-0.987)	0.046	0.402 (0.167-0.969)	0.042
Fusion protein (p190 *vs.* p210 and p190/210)	0.939 (0.582, 1.515)	0.797		
Imatinib *vs.* dasatinib	1.305 (0.807, 2.109)	0.277		
Allogeneic transplant	0.682 (0.362, 1.284)	0.23		

CMR: complete molecular response; OR: odds ratio; 95% CI: 95% confidence interval; DFS: disease-free survival; HR: hazard ratio.

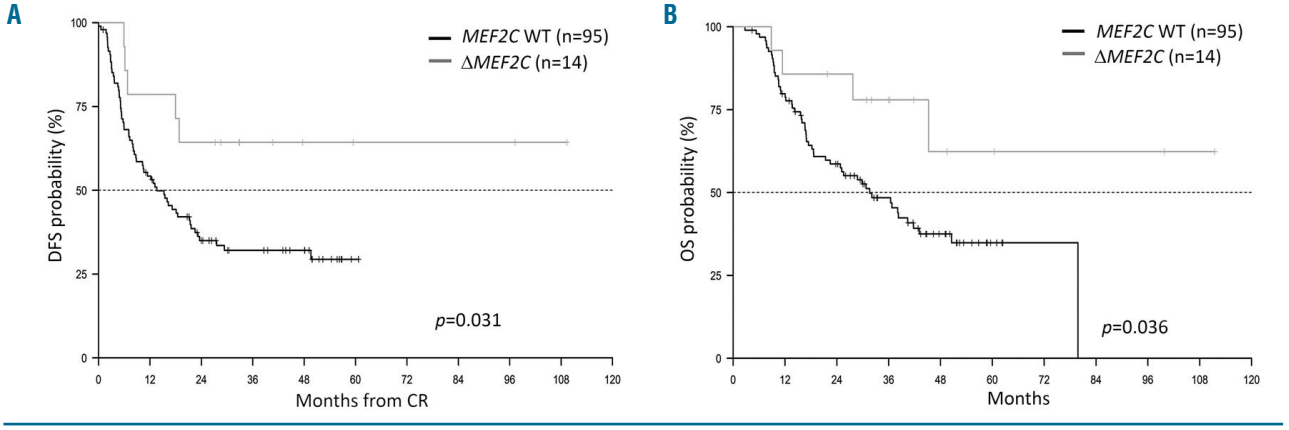

Figure 4. Survival probability curves according to *MEF2C* status. (A) Disease-free survival at 36 months of Δ*MEF2C versus MEF2C* wild-type patients. (B) Overall survival at 36 months of Δ*MEF2C versus MEF2C* wild-type patients. WT: wild-type; DFS: disease-free survival; OS: overall survival; CR: complete remission.

ing patients into high- and low-risk categories are urgently needed to further optimize treatment. Moreover, another unsolved issue is whether all eligible patients should undergo HSCT,[7,17] a procedure still associated with short- and long-term side effects, as well as treatment-related mortality. This is particularly important for patients who obtain a CMR.

To address these issues we sought to identify additional genomic lesions with prognostic significance in adult Ph+ ALL using high density Cytoscan arrays. We found that adult Ph+ ALL patients carried an average of 7.8 aberrations each, with deletions outnumbering gains, in line with other ALL subsets.[22,30,38,39] Macro-aberrations were identified in 48% of cases and micro-aberrations in the majority of patients: among the latter, the most frequent was Δ*IKZF1*, which was detected in 84% of cases. Δ*IKZF1* has been regarded as a poor prognostic marker in both childhood and adult ALL.[34,36,39-41] This finding was not, however, confirmed in our cohort: in fact, the presence of Δ*IKZF1* alone was not associated with a worse DFS. A further evaluation of the various *IKZF1* isoforms showed that only the dominant-negative genotype was deleterious for outcome. In addition, patients with Δ*IKZF1*+*CDKN2A* and/or *PAX5*, accounting for almost half the Δ*IKZF1* cases, experienced a significantly inferior DFS (P=0.005) and overall survival (P=0.02), in line with previous reports on ALL in general.[28,29,36,39,42,43] Δ*IKZF1*+*CDKN2A* and/or *PAX5* also had a prognostic impact in multivariate analysis; survival analysis was carried out merging all cases enrolled in the different trials together in order to gain statistical significance.

Recently, studies have been focused on the presence of additional karyotypic aberrations in Ph+ ALL.[44-48] These studies have highlighted that a high percentage of Ph+ ALL cases (60-80%) harbor additional chromosomal abnormalities, with the most frequent aberrations involving chromosomes 7, 9, and 14. Patients with additional abnormalities, particularly loss of 9/9p and/or *CDKN2A*, have a worse outcome. These results point to the importance of screening for other molecular markers, and not only *IKZF1*, in agreement with our findings on Δ*IKZF1*+*CDKN2A* and/or *PAX5*. At variance from these reports, our study also identified novel lesions that had a favorable impact on outcome. Among these, it is worth mentioning Δ*MEF2C*, which occurred in 18.1% of patients and was of two sizes, a long deletion, encompassing introns 1-2 and exon 2, and a second, smaller one, involving only exon 2. *MEF2C* is a transcription factor involved in B-cell survival and proliferation whose overexpression is associated with an unfavorable prognosis in T-ALL and acute myeloid leukemia.[49-52] In our study, the presence of Δ*MEF2C*-long was associated with achievement of a CMR (P=0.05) and with a significantly better DFS compared to the remaining cases (P=0.031) also in a multivariate model; as for *IKZF1* deletions, survival analysis was performed merging the whole cohort because of the sample sizes. Δ*MEF2C*-long was widely distributed among cases, with no association with white blood cell count, age, type of fusion protein or additional deletions. To our knowledge, this is the first report that correlates Δ*MEF2C*-long with prognosis in Ph+ ALL: Martinelli *et al.*[40] and Mullighan *et al.*[22,41] described Δ*MEF2C* in Ph+ ALL, but did not demonstrate a correlation with outcome. Finally, Δ*KRAS* was associated with a higher rate of CMR achievement upon induction (P=0.01), but not with a better DFS.

In conclusion, we show that additional genetic lesions can be found at presentation in adult Ph+ ALL patients and that these lesions have prognostic significance, with the *IKZF1* dominant-negative isoform and Δ*IKZF1*+*CDKN2A* and/or *PAX5* negatively affecting outcome, and Δ*MEF2C* and Δ*KRAS* being instead associated with a more favorable prognosis. Screening for these genetic lesions should, therefore, be performed at the time of diagnosis for a more refined prognostic stratification, and for a more personalized and tailored management of Ph+ ALL patients.

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