SUPPLEMENTARY APPENDIX

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

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 doi:10.3324/haematol.2018.196055

Received: April 26, 2018.

Accepted: August 30, 2018.

Pre-published: September 6, 2018.

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Supplemental Methods

Description of the study population

Bone marrow and/or peripheral blood samples from 116 patients (56 male and 60 female) with newly diagnosed Ph+ ALL, containing ≥70% of leukemic blasts were analyzed. Median age was 51.1, median white blood cell (WBC) count was 25.4 x 10^9/L, median hemoglobin (Hb) was 9.6 g/dL and platelets count was 50 x 10^9/L. 71 patients were p190 positive, 29 p210 and 16 had both p190 and p210 proteins: the latter 2 groups were considered together for further analyses. All patients provided informed consent in accordance with local ethical committee requirements and the Declaration of Helsinki. The patients evaluated were enrolled in 4 consecutive GIMEMA (Gruppo Italiano Malattie EMatologiche dell'Adulto) clinical trials, specifically designed for adult (LAL0904 3rd emendment[1], LAL1205[2] and LAL1509[3]) and elderly (LAL0201-B[4]) Ph+ ALL cases , and based on a chemo-free induction phase, contemplating the use of a TKI plus steroids; further details are provided in Table S1.

Cytoscan HD Array experiments and data analysis

DNA was extracted using the Wizard® Genomic DNA Purification kit (Promega, Fitchburg, WI) from post-Ficoll mononuclear cells, quantified by Nanodrop and the checked for quality by agarose gel.

Copy number aberrations (CNA) discovery was performed by using Cytoscan HD Array (Affymetrix, Santa Clara, CA). The chips contain more than 2.6 million of markers for copy number analysis and approximately 750000 SNPs that fully genotype the whole genome with a greater than 99% accuracy. Positive (Genomic DNA Control supplied by Affymetrix) and negative controls (Low EDTA TE Buffer) were used to verify the quality of chip, reagents and instruments; furthermore, each CytoScan® HD array has internal quality control metrics used to determine pass/fail for individual samples. Genomic DNA (gDNA, 250 ng) diluted in Low EDTA TE buffer were used to perform CNA experiments and the experiments were performed following the manufacturer's instructions. The procedure included genomic DNA digestion and ligation, PCR amplification, PCR product purification, quantification and fragmentation, labeling, array hybridization, washing and scanning. At the end of each scan, CEL files are created using the GeneChip® System 3000 7G (Affymetrix). The CEL files obtained were analyzed using Chromosome Analysis Suite v2.0 (ChAS) software for the identification of CNAs, as well as for the recognition of minimal common regions of aberration. All the CNAs were visually inspected by dChipSNP software (https://sites.google.com/site/dchipsoft/). Variants reported in the Database of

genomic variants (http://projects.tcag.ca/variation) and/or those detected in germline DNA of patients were excluded from the analysis. Furthermore, all CNAs were compared with two large cohorts of healthy individuals (n=200) belonging to the HapMap project.

MLPA experiments and data analysis

The copy number status of *IKZF1*, *CDKN2A/B*, *PAX5*, *ETV6*, *EBF1* and *BTG1* was confirmed by multiplex ligation-dependent probe amplification (MLPA) using the Salsa MLPA P335 ALL-IKZF1 kit (MRC-Holland, Amsterdam, The Netherlands) and following manufacturer's instructions. This kit contains probes for selected B-cell development and differentiation genes and permits to detect abnormalities with a sensitivity of 20-30%. The full list and location of the MLPA probes are available at the MRC Holland website (http://www.mrc-holland.com) One hundred nanograms of gDNA extracted from bone marrow and/or peripheral blood samples of the patients was used and gDNA extracted from 10 healthy donors was used as wild-type control, at least 3 per experiment.

Amplicons were separated on an ABI-Prism 3130 sequencer and analyzed by GeneMapper 4.0 software (Applied Biosystem, Life Technologies, Foster City, CA).

The detection of abnormal copy number values was performed by Coffalyser.Net software and manufacturer's instructions (www.coffalyser.net). Peak heights <0.7 times the control peak height were considered as deletions and those >1.3 times the control peak height represented duplications.

The same kit was also used for the screening of *IKZF1* isoforms.

Droplet Digital PCR assay

The validation of *MEF2C* (5q14.3) deletions was performed using Droplet Digital PCR (ddPCR; QX200, Bio-Rad, Hercules, CA) System. In our experimental procedure for probe design, it was crucial an accurate identification of minimal common region (MCR) of deletions of *MEF2C*. This was carried out in two separate steps: first, the ChAS software generated a report that includes all the regions affected by gains or losses in each sample; next, a careful analysis was performed by manual curation to identify the MCR detected in all cases. The MCR resulted to be chr5:88122179-88127630, so the probes were designed in this region and marked in FAM fluorochrome (dye).

We choose *ITGA2* (5q11.2) and *EIF2C1* (1p34.3) as reference genes since they were never affected by aberrations in our cohort and they were labeled with HEX dye.

The QX200TM Droplet Digital PCR System was used according to manufacturer's instructions and each sample was amplified in triplicate. The reaction mixtures for ddPCR contained the following components: 40 ng of gDNA, 11 µl 2X ddPCR Supermix (Bio-Rad), 1 µl of each *MEF2C* and

ITGA2 probes, 0.5 µl of *EIF2C1*, in a final volume of 20 µl. This reaction mixture was loaded into the sample well of an eight-channel disposable droplet generator cartridge (DG8 cartridge Bio-Rad), together with 70 µl of droplet generation oil, into the QX200 Droplet Generator. Following droplets' generation, a volume of 40 µl was manually transferred into a 96-well PCR plate and then amplified. The PCR-positive and PCR-negative droplets are counted using the QuantaSoft Analysis Pro v1.0 software to provide an absolute quantification of target DNA. Quality controls including no amplification in non template controls (NTC) wells (2 for each experiment), exclusion of wells with less than 10,000 accepted droplets, fluorescence amplitude of positive and negative droplets, and Poisson mean estimates were checked.

According to the manufacturer's application guide, we considered acceptable only the replicates with a number of droplets ≥10000. Copy number is determined by calculating the ratio of the target molecule concentration to the reference molecule concentration, times the number of copies of reference species in the genome, in our case 2: $\frac{[target]}{[reference]} \times 2$.

Mutational screening

PCR primers for *MEF2C* and *KRAS* coding exon were custom- designed using the Primer 3 online software (http://frodo.wi.mit.edu/primer3/) and the *in silico* PCR tool of the University of California, Santa Cruz (UCSC) Human Genome database (http://genome.ucsc.edu/) to verify the uniqueness of the match (Table S2).

Sanger sequencing for *MEF2C* and *KRAS* mutations was performed with the ABI-Prism 3500 sequencer (Applied Biosystem, Life Technologies, Foster City, CA): sequences were compared with the reference sequence by the Mutation Surveyor Version 3.97 software (SoftGenetics) to identify candidate mutations automatically and by manual curation.

Statistical analysis

Logistic regression models were used in univariate and multivariate analyses to assess the effect of clinical, biologic and genomic factors.

Overall (OS) and disease-free survival (DFS) curves were estimated with the Kaplan-Meier product-limit method and log-rank test. Differences in DFS, were evaluated by Cox regression model in univariate and multivariate analyses. Complete molecular response (CMR) achievement and DFS probability were calculated according to demographic, clinical and biological characteristics and genomic characterization. All significant variables in univariate analysis were

included in multivariate analysis. Analyses were performed using the SAS system software (version 9.4).

Table S1. List of GIMEMA clinical trials.

Protocol ID	Analyzed cases / patients enrolled	NCT number	Ages eligibility	Treatment scheme	End of induction	Allo SCT^
LAL0201B[9]	13/49	NCT00376467	>60 yrs	Imatinib 800mg/day until day 45; consolidation based on medical choice	Day +50	0
LAL0904 3rd amendment[10]	27/51	NCT00458848	15-60 yrs	Imatinib 600mg/day until day 50; consolidation with HAM followed by allo-SCT, if feasible	Day +50	15
LAL1205[11]	37/53	NCT00391989	18 yrs onwards; no upper limit	Dasatinib 70mg twice/day until day 84 day; consolidation based on medical choice	Day +85	6
LAL1509[12]	39/60	NCT01361438	18-60 yrs	Dasatinib 140mg/day until day 84; for CMR: Dasatinib until progression; for non-CMR: clofarabine +endoxan followed by allo-SCT, if feasible	Day +85	14

^Among patients included in the present study, *allo-SCT: allogeneic stem cell transplant

Table S2. Primers used for the detection of *MEF2C* and *KRAS* mutations.

	Target region (hg19)	Forward	Reverse
MEF2C			
Exon 2	chr5:88823592-88823924	CGAATGCAGGAATTTGGGAACT	CAGCACTTTAACTGGTCACATT
Exon 3	chr5:88804519-88804931	GAATGTGTTAGTGCCCAGGG	GTGTATGTGTGTGTGGCAGG
Exon 4	chr5:88761059-88761403	TGAACTTCTTTAATGCCCCTGA	GGGTGAGTGCATAAGAGGAGT
Exon 5	chr5:88751776-88752115	GGCTGTCTGTCTTGAAGAGC	TCGTAGATAAAGCAGTGTTGGC
Exon 6	chr5:88748836-88749176	AACGTTTGAGCACAGCATGG	TGTCCTGCAAATCACCTAGTAGA
Exon 7	chr5:88731597-88732019	GGTTTTGCAATGTACGTCTTACC	CTCTGTCAATGGCACTGTTATGT
Exon 8	chr5:88730055-88730378	GGAGTTATCTTTGGAGTCTGGG	ACCTGTGAGTGATGCCAGAA
Exon 9	chr5:88729111-88729500	GGGTTGGCAGAGTTCATAGAG	CGCAGGCCCTAAATAAAGCT
Exon 10	chr5:88728399-88728772	TCTCCCCTCCCCTCAAAATT	CCGTTAATGGGATATTGAAGCAC
Exon 11	chr5:88722545-88723031	GCTCTGGTGTCTATGCGAGT	AGGTATAGCACACACACACAC
KRAS			
Exon 2	chr12:25398064-25398554	TCTTAAGCGTCGATGGAGGAG	TTGAAACCCAAGGTACATTTCAG
Exon 3	chr12:25380085-25380490	CGTCATCTTTGGAGCAGGAAC	ATGCATGGCATTAGCAAAGAC
Exon 4	chr12:25378389-25378834	TGGTGTAGTGGAAACTAGGAATTACAT	TGGATTAAGAAGCAATGCCCT

Table S3. Frequency and description of macroaberrations.

Chromosome	Region involved	Type of CNA	Number of patients (%)
1	Whole long arm	Gain	9 (7.7)
2	Whole chromosome	Gain	4 (3.4)
3	Whole short arm	Loss	4 (3.4)
4	Whole chromosome	Gain	4 (3.4)
5	Whole chromosome	Gain	2 (1.7)
6	Whole chromosome	Gain	3 (2.6)
7	Whole chromosome/arm	Loss	21 (18.1)
8	Whole chromosome	Gain	3 (2.6)
8	Whole short arm	Loss	1 (0.8)
9	Whole chromosome/arm	Loss	11 (9)
9	Whole chromosome	Gain	1 (0.8)
10	Whole chromosome	Gain	2 (1.7)
11	Whole chromosome	Gain	1 (0.8)
12	Whole short arm	Loss	3 (2.6)
13	Whole long arm	Gain	3 (2.6)
14	Whole chromosome/arm	Gain	7 (6)
15	Whole chromosome	Loss	1 (0.8)
16	Whole long arm	Loss	1 (0.8)
16	Whole chromosome	Gain	1 (0.8)
17	Whole chromosome	Gain	2 (1.7)
18	Whole chromosome	Gain	1 (0.8)
19	Whole chromosome	Gain	1 (0.8)
20	Whole long arm	Loss	3 (2.6)
20	Whole chromosome	Gain	1 (0.8)
21	Whole long arm	Gain	3 (2.6)
22	Whole long arm	Gain	2 (1.7)

Table S4. Detail of the recurrently deleted novel genes.

	FOCAD	*CDK6*	*PTPRD*	*MEF2C*	*JAK2*	*ADD3*	*SLX4IP*	*HBS1L*	*ATP10A*	*KRAS*	*ARHGAP2*
pt.1	chr9:203861-38481228		chr9:203861-38481228		chr9:203861-38481228						
pt.2	chr9:203861-37336325	chr7:43360-159119707	chr9:203861-37336325		chr9:203861-37336325						
pt.3	chr9:402293-67983174		chr9:402293-67983174		chr9:402293-67983174						
pt.4	chr9:203861-37829117		chr9:203861-37829117		chr9:203861-37829117						
pt.5	chr9:203861-68342770	chr7:43360-159119707	chr9:203861-68342770		chr9:203861-68342770						
pt.6	chr9:203861-38330977		chr9:203861-38330977		chr9:203861-38330977						
pt.7	chr9:203861-38771831		chr9:203861-38771831		chr9:203861-38771831						
pt.8	chr9:203861-67983174		chr9:203861-67983174		chr9:203861-67983174	chr10:111768930-111868135					
pt.9	chr9:203861-38776786		chr9:203861-38776786		chr9:203861-38776786		chr20:10417444-10502474		chr15:26035609-26113711		
pt.10	chr9:20485438-20759956	chr7:92317751-92490774	chr9:8050902-8903857		chr9:5123013-5234403	chr10:111768930-111868135	chr20:10407144-10460323				
pt.11	chr9:203861-38771831		chr9:203861-38771831		chr9:203861-38771831						
pt.12							chr20:10417414-10452297		chr15:26036071-26103200	chr12:25402194-25538043	
pt.13							chr20:10417444-10451891				
pt.14		chr7:43360-159077223									
pt.15				chr5:88117376-88127630		chr10:111768930-111868135		chr6:135373270-135438522			
pt.16	chr9:20616622-38771774										
pt.17				chr5:88111711-88128409				chr6:135365131-135441914			
pt.18								chr6:135373270-135438522			
pt.19		chr7:63156201-155464016						chr6:135373270-135438457			
pt.20	chr9:20626602-36928914										
pt.21										chr12:8594880-32188752	
pt.22										chr12:173786-25613822	

pt.23							chr20:10417444-10460323				
pt.24				chr5:88103154-88189524						chr12:25402194-25538043	
pt.25						chr10:111768930-111868135					
pt.26						chr10:111768930-111868135					
pt.27										chr12:173786-26491475	chr4:86490169-86615490
pt.28										chr12:25401502-25537468	chr4:86490169-86615490
pt.29				chr5:88118171-88416354					chr15:26036071-26113711		
pt.30	chr9:203861-71515539		chr9:203861-71515539		chr9:203861-71515539						
pt.31	chr9:19478278-22657330	chr7:57970785-137552904									
pt.32	chr9:20626602-36983794										
pt.33			chr9:8153932-8854489						chr15:25978811-26113711		
pt.34	chr9:203861-68342770		chr9:203861-68342770		chr9:203861-68342770						
pt.35	chr9:20246757-23771094										
pt.36		chr7:57970785-122470586	chr9:203861-12323745	chr5:88118171-88127630	chr9:203861-12323745	chr10:111768930-111868135			chr15:26036071-26113711		
pt.37	chr9:203861-133678014	chr7:92311479-92467722	chr9:203861-133678014		chr9:203861-133678014	chr10:111768930-111868135					chr4:86493637-86601680
pt.38	chr9:203861-68330127	chr7:57960621-159119707	chr9:203861-68330127		chr9:203861-68330127						
pt.39	chr9:20685149-38776786									chr12:25402194-25545315	
pt.40	chr9:20549838-26867216										
pt.41	chr9:203861-38771831		chr9:203861-38771831		chr9:203861-38771831						
pt.42	chr9:13875756-38772005	chr7:63253896-151905276									
pt.43	chr9:19410341-38787480			chr5:87976675-88990208							
pt.44	chr9:19076865-29811164						chr20:10414642-10517962		chr15:26015279-26103200		chr4:86395830-86436188
pt.45	chr9:203861-68262792		chr9:203861-68262792		chr9:203861-68262792		chr20:10417444-10451891		chr15:25994633-26113711		
pt.46	chr9:203861-37184995		chr9:203861-37184995		chr9:203861-37184995						

pt.47		chr7:63156201-145914853									
pt.48	chr9:203861-67983174		chr9:203861-67983174		chr9:203861-67983174	chr10:111768930-111868135	chr20:10417444-10459613				chr4:86493655-86595882
pt.49									chr15:26053071-26113711		
pt.50									chr15:26055568-26113711		
pt.51						chr10:111768930-111868135			chr15:26036071-26103185		
pt.52						chr10:111768930-111868135			chr15:26036071-26103200		
pt.53						chr10:111768930-111868135	chr20:10407144-10452297		chr15:26036071-26113711		chr4:86493655-86532432
pt.54									chr15:26036071-26103185		
pt.55								chr6:135373270-135438522	chr15:25953197-26113711		
pt.56						chr10:111768930-111856014	chr20:10417444-10451891				chr4:86493655-86532432
pt.57				chr5:88118171-88128409			chr20:10417444-10459613				
pt.58						chr10:111795029-111868135	chr20:10417444-10452297				
pt.59						chr10:111768930-111868135	chr20:10407144-10460323				
pt.60						chr10:105769701-115414911	chr20:10417414-10460323				
pt.61						chr10:111768930-111868135	chr20:10417444-10460323				
pt.62							chr20:10417402-10460323				
pt.63							chr20:10417444-10451891				
pt.64		chr7:44817005-159119707		chr5:88118136-88173694							
pt.65		chr7:43360-159119707									
pt.66		chr7:57970785-143218955									
pt.67		chr7:43360-159119707									
pt.68		chr7:92220800-92496875									
pt.69		chr7:57974597-159119707									
pt.70		chr7:71475429-145941361									

pt.71		chr7:83095813-102108193		chr5:88122179-88176107				chr6:135373270-135438522			
pt.72		chr7:91373218-95916539									
pt.73		chr7:91467414-92675838		chr5:88118171-88153874							
pt.74		chr7:92309320-92467670									
pt.75		chr7:92234736-92462234									
pt.76		chr7:106958-158989411									
pt.77				chr5:88118136-88165607				chr6:135373270-135438457			
pt.78						chr10:111768930-111868135					
pt.79				chr5:88118717-88178552				chr6:135373270-135438522			
pt.80								chr6:135353850-135438522			
pt.81								chr6:135373270-135418257			
pt.82				chr5:88118171-88127630				chr6:135375338-135438522			
pt.83								chr6:135373270-135438522			
pt.84				chr5:88121187-88173736				chr6:135373270-135438522			
pt.85								chr6:135373270-135438522			
pt.86								chr6:135373270-135438522			
pt.87				chr5:88118171-88173759				chr6:135369912-135435171			
pt.88						chr10:111768930-111853667					
pt.89				chr5:88122179-89094716							chr4:86490169-86615490
pt.90				chr5:88117376-88212908							
pt.91				chr5:88118171-88307981							
pt.92				chr5:88118171-88128409							
pt.93				chr5:88118171-88176367							
pt.94				chr5:88111162-88128409							

References

1. Chiaretti S, Vitale A, Vignetti M, et al. A sequential approach with imatinib, chemotherapy and transplant for adult Ph+ acute lymphoblastic leukemia. Final results of the GIMEMA LAL 0904 study. Haematologica. 2016; 101(12):1544-1552.
2. Foà R, Vitale A, Vignetti M, et al. Dasatinib as first-line treatment for adult patients with Philadelphia chromosome-positive acute lymphoblastic leukemia. Blood. 2011;118(25):6521-8.
3. Chiaretti S, Vitale A, Elia L, et al. Multicenter Total Therapy Gimema LAL 1509 Protocol for De Novo Adult Ph+ Acute Lymphoblastic Leukemia (ALL) Patients. Updated Results and Refined Genetic-Based Prognostic Stratification. ASH Annual Meeting Abstracts 2015; 126(23):81.
4. Vignetti M, Fazi P, Cimino G, et al. Imatinib plus steroids induces complete remissions and prolonged survival in elderly Philadelphia chromosome-positive patients with acute lymphoblastic leukemia without additional chemotherapy: results of the Gruppo Italiano Malattie Ematologiche dell'Adulto (GIMEMA) LAL0201-B protocol. Blood. 2007;109(9):3676-8.