SUPPLEMENTARY APPENDIX

Targeted sequencing identifies associations between IL7R-JAK mutations and epigenetic modulators in T-cell acute lymphoblastic leukemia

Carmen Vicente,[1,2] Claire Schwab,[3] Michaël Broux,[1,2] Ellen Geerdens,[1,2] Sandrine Degryse,[1,2] Sofie Demeyer,[1,2] Idoya Lahortiga,[1,2] Alannah Elliott,[3] Lucy Chilton,[3] Roberta La Starza,[4] Cristina Mecucci,[4] Peter Vandenberghe,[1] Nicholas Goulden,[5] Ajay Vora,[6] Anthony V. Moorman,[3] Jean Soulier,[7] Christine J. Harrison,[3,8] Emmanuelle Clappier,[7,8] and Jan Cools[1,2,8]

[1]Center for Human Genetics, KU Leuven, Belgium; [2]Center for the Biology of Disease, VIB, Leuven, Belgium; [3]Leukaemia Research Cytogenetics Group, Northern Institute for Cancer Research, Newcastle University, Newcastle-upon-Tyne, UK; [4]Hematology Unit, University of Perugia, Polo Unico S.M. Misericordia, Italy; [5]Department of Haematology, Great Ormond Street Hospital, London, UK; [6]Department of Haematology, Sheffield Children's Hospital, Sheffield, UK; and [7]U944 INSERM and Hematology Laboratory, St-Louis Hospital, APHP, Hematology University Institute, University Paris-Diderot, Paris, France

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Correspondence: jan.cools@cme.vib-kuleuven.be, christine.harrison@newcastle.ac.uk, emmanuelle.clappier@sls.aphp.fr

SUPPLEMENTARY INFORMATION

Targeted sequencing identifies association between IL7R-JAK mutations and epigenetic modulators in T-cell acute lymphoblastic leukemia

Carmen Vicente[1,2], Claire Schwab[3], Ellen Geerdens[1,2], Michaël Broux[1,2], Sandrine Degryse[1,2], Sofie Demeyer[1,2], Idoya Lahortiga[1,2], Alannah Elliott[3], Lucy Chilton[3], Roberta La Starza[4], Cristina Mecucci[4], Peter Vandenberghe[1], Nicholas Goulden[5], Ajay Vora[6], Anthony V. Moorman[3], Jean Soulier[7], Christine J. Harrison[3,8], Emmanuelle Clappier[7,8], Jan Cools[1,2,8]

[8]Corresponding authors:
Jan Cools (jan.cools@cme.vib-kuleuven.be),
Christine Harrison (christine.harrison@newcastle.ac.uk),
Emmanuelle Clappier (emmanuelle.clappier@sls.aphp.fr)

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The following supplementary files are available as a separate .xls file:

Supplementary tables 1, 2, 3 and 4

1. Supplementary Methods

DNA samples

T-ALL patients (n=155: 111 children, 44 adults) were collected from various institutions. Samples had been previously characterized for oncogene expression and classified into oncogenic subgroups according to combined FISH (fluorescence in situ hybridization) analyses, immunophenotypic data and oncogene expression. Copy number changes of *CDKN2A* (n=95), *MYB* (n=115) and *PTPN2* (n=80) had been previously identified by FISH, MLPA (Multiplex Ligation-dependent Probe Amplification) or arrayCGH. The *HOXA*+, *TLX1*+ and *TLX3*+ and *TAL1/LMO2*+ groups were based on the presence of *HOXA*, *TLX1*, *TLX3*, *TAL1* or *LMO2* rearrangements or by having a *HOXA*, *TLX1*, *TLX3* or *TAL1/LMO2* expression signature. The immature T-ALL group (also known as early thymic or T-cell precursor T-ALL) was defined immunophenotypically or based on gene expression cluster analysis.

Haloplex library preparation and sequencing

Enrichment of the region of interest was performed using the Haloplex Target Enrichment System-Fast Protocol. Briefly, 200 ng of DNA per sample was aliquoted into 8 digestion reactions, each containing 2 restriction enzymes. DNA from the 8 reactions was then pooled and hybridized to Haloplex probes, allowing for purification using magnetic beads. Purified fragments were ligated, amplified and barcoded through 19 cycles of PCR and samples were sequenced on a HiSeq2000 instrument using a 100 bp paired-end protocol. The HiSeq Paired End Cluster Generation Kit was used to generate the clusters and the TruSeq SBS Kit v3 was used for sequencing. Image analysis and base calling was performed using the Illumina RTA software version 1.13.48.

The additional custom filtering criteria refers to the fact that we excluded five variants that were identified in nearly all samples analyzed. Since those variants were not found in dbSNP138, 1000 Genomes or the COSMIC databases, we believe these are false positives of data analysis. These are the variants that have been excluded from the analyses:

Position	Gene	Nucleotide change	Amino acid change
6037058	PMS2	delAA	
103141302	RELN	C>CT	2853G>GS
90645514	IDH2	G>GT	37R>SR
29508805	NF1	T>GT	Splice
103629804	RELN	insGCCGCC	Splice

Validation of identified genomic lesions

We sequenced 158 selected variants (**Supplementary Table 3**) using Sanger sequencing. Analysis of the chromatograms from Sanger sequencing was performed using CLC Main Workbench 6 (CLC Bio).

Western blot analyses

Cells were lysed in cold lysis buffer containing 5 mM Na_3VO_4 and protease inhibitors (Complete tablets, Roche). The proteins were separated on NuPAGE NOVEX Bis-Tris 4%–12% gels (Invitrogen) and transferred to PVDF membranes. Subsequent Western blot analysis was performed using primary antibodies directed against JAK1 (Millipore), JAK3, phospho-STAT5, (Cell Signaling), phospho-JAK1, STAT5 (Santa Cruz Biotech.) and β-actin (Sigma). Anti-phospho-JAK1 antibody was used to detect both phosphorylated JAK1 and JAK3. Western blot detection was performed using secondary antibodies conjugated with horseradish peroxidase (GE Healthcare) and western blot lightning plus-ECL (PerkinElmer).

Statistical analyses

Statistical analyses were carried out using SPSSstatistics v21. Pearson's X^2 Fisher's exact tests were performed to test significance levels for nominal data distributions, whereas the Mann-Whitney U test was used for continuous data.

Validation of the newly identified FLT3 S471C mutation

The FLT3-S471C variant was generated by GenScript, and was cloned into the MSCV-GFP vector. Viral vector production, retroviral transduction and culture of Ba/F3 cells were performed as previously described.[1] Ba/F3 cells expressing the FLT3-ITD (W51 mutation, Kelly et al.[2]) and FLT3-D835Y were previously described.[3] Western blot analyses were performed as indicated above using antibodies directed against FLT3, STAT5 (Santa Cruz Biotech.), anti-phosphoFLT3 (Tyr591), phospho-STAT5 (Cell Signaling) and β-actin (Sigma). Immunoprecipitations was not

performed prior western blot analyses. In order to test the sensitivity of the FLT3-S471C variant to FLT3 inhibition, Ba/F3 cells expressing FLT3-S471C, FLT3-ITD and FLT3-D835Y were treated with 1000nM of the inhibitor AC220 for 30 minutes, and analyzed by western blotting. For dose-response experiments we used 8 different AC220 concentrations (50, 100, 200, 400, 800, 1600, 3200 and 6400 nM) each in triplicate with DMSO as a negative control. The number of viable cells was counted after the treatment 24h with ATP-lite 1step reagent (PerkinElmer). Luminescence was measured with the multilabel plate readers Envision and Victor X4 (PerkinElmer). IC50 values were calculated with GraphPad Prism.

2. Supplementary Results

In our association analyses, the putative *JAK3* passenger mutants (JAK3 Q283H and Q865E mutants that were unable to transform Ba/F3 cells) were initially considered as mutants. We performed the same association analyses excluding those JAK3 passenger mutant cases (scored now as wild type).

Then, the frequency of cases with *IL7R-JAK* mutations in our series is 26.5% (instead 27.7%). All the associations we found with the IL7R-JAK positive cases remained significant as shown in the table below.

	IL7R-JAK signaling pathway			
	Wt (n=114)	**Mut (n=41)**	***P***	**Type of Association**
Clinical				
Gender			0.539	
Male	85 (74.6%)	28 (68.3%)		
Female	29 (25.4%)	13 (31.7%)		
Median age (range)	11 (1-63)	12 (2-66)	0.849†	
T-ALL clusters	**Wt**	**Mut**	***P***	
HOXA+ (n=15)	6 (5.3%)	9 (22%)	**0.004‡**	Positive
TLX1+ (n=13)	10 (11.0%)	3 (10.3%)	1.000‡	
TLX3+ (n=28)	15 (13.3%)	13 (32.5%)	**0.007**	Positive
TAL1/LMO2+ (n=54)	49 (43.4%)	5 (12.2%)	**<0.001**	Negative
Immature+ (n=14)	4 (4.3%)	10 (25.6%)	**0.001‡**	Positive
Del9p21 status	**Wt**	**Mut**	***P***	
Wild-type	28 (24.6%)	12 (29.3%)		
Mutant	86 (75.4%)	29 (70.7%)	0.541	
PHF6 status	**Wt**	**Mut**	***P***	
Wild-type	99 (86.8%)	26 (63.4%)		
Mutant	15 (13.2%)	15 (36.6%)	**0.001**	Positive
PRC2 status	**Wt**	**Mut**	***P***	
Wild-type	99 (86.8%)	29 (70.7%)		
Mutant	15 (13.2%)	12 (29.3%)	**0.020**	Positive
WT1 status	**Wt**	**Mut**	***P***	
Wild-type	104 (91.2%)	30 (68.3%)		
Mutant	10 (8.8%)	13 (31.7%)	**0.001**	Positive

3. Supplementary Figures

Figure S1. The newly identified mutation in the extracellular domain of FLT3 (S471C) is a transforming mutation

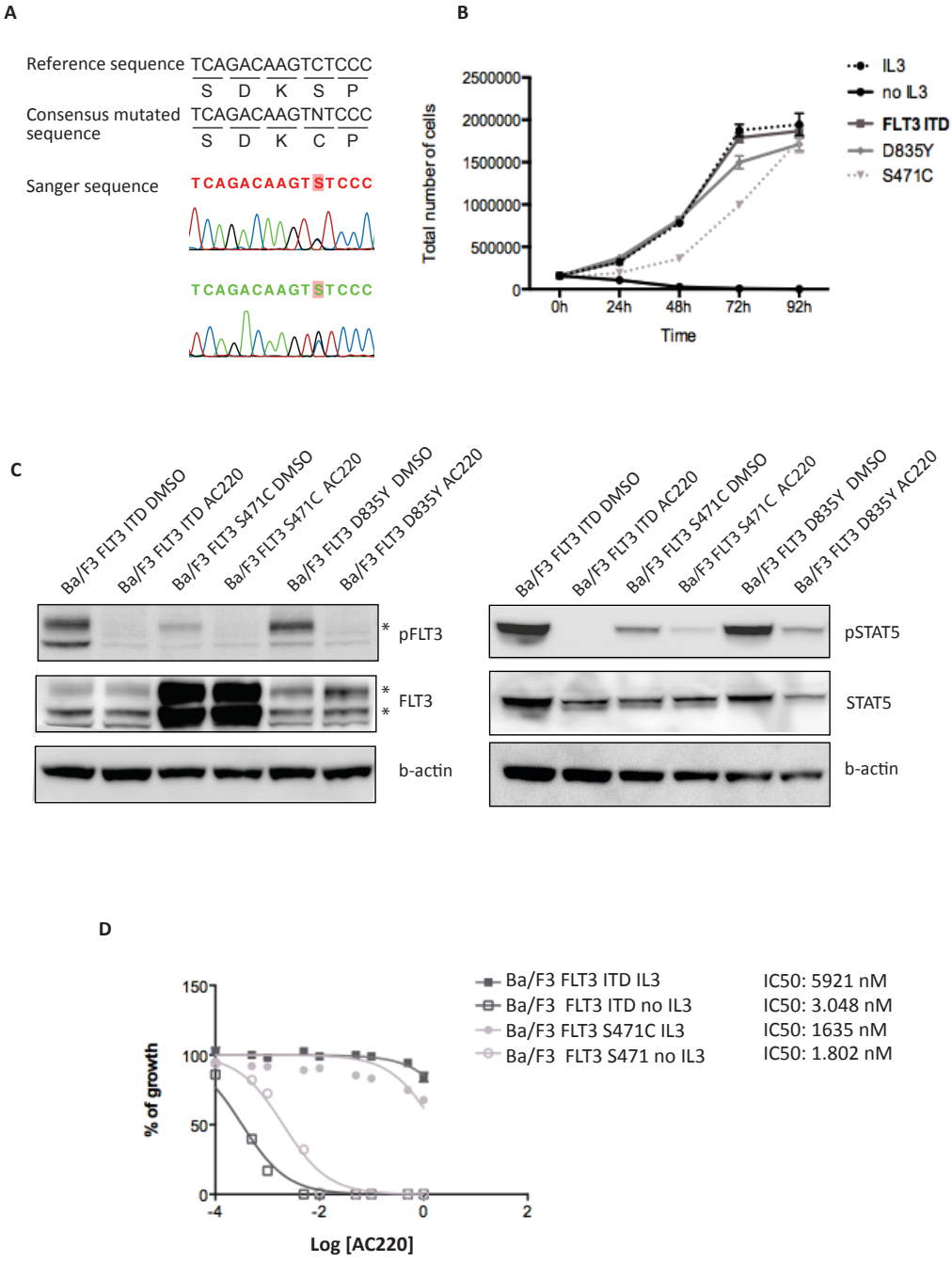

Figure S1. The newly identified mutation in the extracellular domain of FLT3 (S471C) is a transforming mutation. (A) Sanger sequencing confirmation of the FLT3-S471C variant **(B)** Proliferation curve of Ba/F3 cells expressing FLT3-ITD, FLT3-D835Y and FLT3-S471C variants. Cells expressing FLT3-S471C were able to grow in the absence of IL3. Ba/F3 cells expressing FLT3-ITD, FLT3-D835Y or Ba/F3 wild type supplemented with IL-3 were used as a positive control. Cells expressing Ba/F3 cells wild type without IL-3 were used as a negative control. **(C)** Western blotting showing the phosphorylation and expression of signaling proteins. The expression in Ba/F3 cells of the variant FLT3-S471C resulted in constitutive phosphorylation of FLT3 and STAT5. The level of phosphorylation was lower compared to Ba/F3 cells carrying FLT3-ITD and FLT3-D835Y that correlates with the slower proliferation detected also in comparison with cells expressing FLT3-ITD or FLT3-D835Y. The phosphorylation of FLT3 was reduced for all the Ba/F3 upon treatment with AC220. However, the phosphorylation of STAT5 could be detected after the treatment with AC220 for the cells carrying the S471S and the D835Y mutations. **(D)** We calculated the IC50 values for the AC220 inhibitor treating the cells with FLT3-S741C or FLT3-ITD with increasing concentrations of the inhibitor and measuring the proliferation after 24 hours of inhibition. Both cell lines were highly sensitive to the treatment with AC220 with IC50 values lower than 5 nM.

4. Additional references

1. Degryse S, de Bock CE, Cox L, et al. JAK3 mutants transform hematopoietic cells through JAK1 activation causing T-cell acute lymphoblastic leukemia in a bone marrow transplant mouse model. *Blood*. 2014.
2. Kelly LM, Liu Q, Kutok JL, et al. FLT3 internal tandem duplication mutations associated with human acute myeloid leukemias induce myeloproliferative disease in a murine bone marrow transplant model. *Blood*. 2002;99(1):310–318.
3. Pauwels D, Sweron B, Cools J. The N676D and G697R mutations in the kinase domain of FLT3 confer resistance to the inhibitor AC220. *Haematologica*. 2012;97(11):1773–1774.