



TO THE EDITOR:

Caution encouraged in next-generation sequencing immunogenetic analyses in acute lymphoblastic leukemia

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We read with interest the report of whole (epi)genome analyses in 8-month-old monozygotic twins with pro-B CD10⁺ “B other” B-cell precursor (BCP)-acute lymphoblastic leukemia (ALL) that described a common *TCF3-ZNF384* rearrangement and distinct converging *PTPN11* mutations.¹

Bueno et al¹ also concluded that the initial prenatal oncogenic event(s) arose in an early hematopoietic fetal pre-B-cell receptor (BCR) progenitor or stem cell, rather than in a prehematopoietic precursor, because the shared and the individual leukemia-specific somatic mutations were not found in cultured bone marrow-derived mesenchymal stem cells and clonal immunoglobulin (Ig) variable diversity joining (VDJ) rearrangements were not identified in leukemic blasts from either twin. We contest this conclusion for 2 reasons.

First, the complex rearrangement leading to the *TCF3-ZNF384* fusion resulted from a 3-way t(12;14) and t(14;19) translocation, with chromosome 14 breakpoints compatible with involvement of the IgH locus, which is well recognized in BCP-ALL.² This would incapacitate 1 of the 2 IgH alleles and place the initial oncogenic event after the start of IgH rearrangement or, at least, accessibility of the IgH locus.

Second, as shown in supplemental Table 1 in their article, both twins were followed by allele-specific IgH minimal residual disease quantification. This was performed in 1 of our laboratories, using standardized EuroMRD allele-specific quantification of IgH VDJ rearrangements identified at diagnosis. Next-generation amplicon sequencing of IgH VDJ and diversity-joining (DJ) rearrangements from DNA showed clear evidence of clonal IgH VDJ rearrangements with 2 independent bioinformatic pipelines³⁻⁵: the most frequent clonotypes corresponded to 42% of IgH VDJ reads in Twin A and 90% of IgH VDJ reads in Twin B (Table 1).

On closer analysis of the most frequent clonotypes, we identified a shared incomplete DJ rearrangement that was present in the major clone from both twins, as well as in 1 to 3 other significant clonotypes with DJ or VDJ rearrangement, corresponding to 1% to 4% of IgH VDJ reads in both twins and 8% of IgH DJ reads (Twin A only). Of note, this common initial rearrangement did not have any N nucleotide additions, which

is suggestive of a rearrangement prior to the onset of DNA nucleotidyl exotransferase activity. All VDJ rearrangements were out-of-frame, suggesting a selection against in-frame rearrangements in this shared leukemia. The second most frequent VDJ rearrangement in Twin A did not share the same DH segment and had N additions, in keeping with a distinct evolution from a common pre-DJ precursor rather than the other IgH allele, if the second IgH allele was involved in the 3-way *TCF3-ZNF384* translocation.

This clearly places the cell of origin (COO) around the onset of bone marrow Ig DJ rearrangement, with ongoing VDJ rearrangement, in keeping with the conclusions of Bueno et al.¹ Ongoing VDJ rearrangement is well recognized in BCP-ALL.⁶

However, it is very surprising that these IgH VDJ rearrangements were not identified by the BCR analyses cited in their supplemental data. The difference appears to be due to the fact that out-of-frame Ig rearrangements were filtered out from Ig repertoires analyzed from complementary DNA, thus limiting analysis to in-frame transcribed repertoires. Although such a bioinformatics approach is entirely appropriate for immunogenetics analysis of functional mature B lymphoid repertoires, it is not appropriate for analysis of BCP-ALL, when most rearrangements are out-of-frame,⁷ as was the case here.

To establish the incidence of in-frame Ig VDJ rearrangements in pediatric BCP-ALL, we analyzed the 10 most frequent IgH VDJ and DJ clonotypes from 177 children with BCP-ALL, aged younger than 10 years, using EuroClonality amplicon next-generation sequencing identification of IgH (VDJ and DJ), IgK (kappa deleting element), and T-cell receptor (TR)D targets from diagnostic DNA, with the Vidjil pipeline.⁴ Among a total of 1645 clonotypes (after filtering out germline amplification of IGHD7-27/J1*01 [n = 106], 13 irregularly analyzed TR clonotypes, and 6 atypical Ig clonotypes), 28% were IgH VDJ, 25% were IgH DJ, 19% were IgK, and 29% were TRD (Table 2). Clonotypes representing ≥10% of reads from a given locus were (arbitrarily) considered to have a leukemic origin, and less frequent clonotypes were considered to be reactive. Of 686 leukemic clonotypes, 35% were IgH VDJ, 9.5% were IgH DJ, 16.5% were IgK, and 39% were TRD. Among reactive clonotypes, 22% were IgH VDJ, 35% were IgH DJ, 20% were IgK, and only 22% were TRD,

Table 1. IgH clonotypes identified in diagnostic twin leukemic DNA

Ig CDR3	Locus reads (%)	IF/OF
Twin A: peripheral blood 80% blasts (total 115 000 IgH VDJ and 120 000 DJ reads)		
IGHV3-9*01 0/TAAGGGG/5 IGHD6-13*01 5//5 IGHJ4*02	42	OF*
IGHV3-33*01 4/TC/0 IGHD7-27*01 0/CCTAGT/0 IGHJ4*02	23	OF
IGHV3-9*01 8/CTTTGG/5 IGHJ6*02	12	OF
IGHV3-21*01 0/TCCGCCG/5 IGHD6-13*01 5//5 IGHJ4*02	4	OF
IGHV3-13*01 1/GGAAGG/4 IGHJ5*02	1	OF
IGHV3-74*02 0/AGGGCACGC/3 IGHD6-13*01 5//5 IGHJ4*02	1	OF
Smaller IgH VDJ and filtered reads	16	
D2-2*01 5/20/5 IGHD6-13*01 5//5 IGHJ4*02	8	
Twin B: bone marrow 55% blasts (total 141 000 IgH VDJ and 133 000 DJ reads)		
IGHV3-74*02 2/CCCGTGGG/6 IGHD6-13*01 5//5 IGHJ4*02	90	OF*
IGHV3-30*04 0/TCCCCCTCC/2 IGHD6-13*01 5//5 IGHJ4*02	3	OF
Smaller IgH VDJ and filtered	7	
No frequent DJ with common IGHD6-13*01 5//5 IGHJ4*02 trunk		

The most frequent IgH clonotypes and their relative frequency, as a percentage of total reads for the given locus, are shown. The shared IGHD6-J4 common stem rearrangement is set italic. IF, in-frame; OF, out-of-frame

*CDR3 targets for minimal residual disease quantification by quantitative polymerase chain reaction.

in keeping with deletion of the TRD locus in T lymphocytes undergoing TRA VJ rearrangement.

The number of leukemic clonotypes per patient ranged from 1 to 10. IgH VDJ rearrangements were classified using the IMGT/V-QUEST pipeline as potentially productive or nonproductive, based on maintenance of the CDR3 reading frame, maintenance of the CYS104 amino acid, and the absence of stop codons.^{8,9} BCP-ALL (including Twins A and B) was then classified into cases with ≥ 1 potentially productive leukemic IgH clonotype (even if less frequent than the predominant Ig/TR leukemic clonotypes), those with only nonproductive IgH VDJ clonotypes, those

with only IgH DJ clonotypes (with or without IgK and/or TRD clonotypes), those with no IgH leukemic clonotypes (only IgK and/or TRD), and those with no Ig clonotypes (only TRD). As shown in Table 2, only 27% of 177 cases of pediatric BCP-ALL exhibited productive IgH VDJ rearrangements (or 29% of the 163 cases of BCP-ALL with ≥ 1 IgH VDJ and/or DJ leukemic clonotype), and only 21% (51/243) of IgH VDJ leukemic clonotypes were potentially productive. This was significantly lower than the proportion of productive low-frequency (but still top 10 most frequent) reactive clonotypes (41% or 87/213, $\chi^2 = 21.2$, $P < 10^{-4}$), in keeping with the fact that there is no known evidence for selection of productive IgH rearrangements in BCP-ALL. The

Table 2. Predominant Ig and TR clonotypes in pediatric BCP-ALL

Ig/TR alleles	10 Predominant clonotypes	Leukemic clonotypes*	Reactive clonotypet
Total, N/n	1645‡	686	959
IgH VDJ total	456 (27.7)	243 (35.4)	213 (22.2)
Productive, %	30.7	20.9§	40.8¶
IgH DJ	404 (24.6)	65 (9.5)	339 (35.3)
IgK	309 (18.8)	113 (16.5)	196 (20.4)
TRD	476 (28.9)	265 (38.6)	211 (22)
Total patients, N	177		
≥ 1 productive IgH VDJ	48 (27.1)		
Only nonproductive IgH VDJ	110 (62.2)		
No IgH VDJ but ≥ 1 IgH DJ +/- IgK/TRD	5 (2.8)		
No IgH but ≥ 1 IgK with or without TRD	9 (5.1)		
No Ig but ≥ 1 TRD	5 (2.8)		

The 10 most frequent Ig and TR clonotypes are indicated for 177 patients with BCP-ALL aged younger than 10 years. Unless otherwise noted, data are n (%).

*More than 10% of reads for a given target.

†Less than 10% of reads for a given target.

‡A total of 106 germline IGHD7-27/J1*1, 13 TRA/B/G, and 6 atypical IgH were filtered out.

§Forty of 201 (20%) clonotypes corresponding to $>20\%$ of reads for a given target vs 13 of 42 (31%) of clonotypes corresponding to 10% to 20% of reads for a given target.

¶Fifty-nine of 138 (43%) clonotypes corresponding to 1% to 10% of reads for a given target.

incidence of pediatric BCP-ALL with ≥ 1 productive IgH VDJ allele (30%) is not significantly ($P > .2$) lower than the incidence (19/53) identified in adult patients with BCP-ALL with an IgH DJ rearrangement on ≥ 1 allele.⁷ Restriction of this analysis to the 12 children aged younger than 1 year, including the twins in the study by Bueno et al,¹ revealed that all had IgH VDJ clonotypes, with 5 (42%) demonstrating ≥ 1 in-frame VDJ, a higher incidence compared with children between 1 and 9 years of age (26%).

Taken together, these data confirm that 89% of pediatric BCP-ALLs have detectable IgH VDJ BCR clonotypes at diagnosis when not filtered for productive rearrangements, when the incidence of patients with ≥ 1 leukemic clonotype falls to 27% and the overall incidence of leukemic clonotypes decreases to, at most, 21%. Based on these data, the polyclonal BCR repertoires described by Bueno et al¹ are likely to correspond to non-leukemic Ig VDJ rearrangements, which are well described on NGS analysis of BCP-ALL at diagnosis.¹⁰ The investigators conclude that failure to identify clonal rearrangement reflects a pre-VDJ COO and, by extrapolation, maturation arrest, whereas our data demonstrate that the COO is indeed at the time of early DJ rearrangement, with ongoing VDJ rearrangement. They also demonstrate that the Ig repertoire analytical approach and bioinformatics filtering used failed to describe all major leukemic VDJ clones in both twins.

Increasing immunogenetics bioinformatics sophistication has vastly improved our capacity to understand normal and pathological immune repertoires, but the interpretation of the data generated needs to be performed with knowledge of the underlying pathology and appropriate data filtering. It is also important that the current trend for massive data shifting to supplemental files does not complicate full analysis of the data presented.

Authorship

Contribution: C.A., S.K., and E.M. analyzed results and wrote the manuscript; F.T. developed the Vidjil bioinformatics pipeline and extracted data; J.V., M.S., and A.P. contributed patient samples and data; and M.B. critically appraised the data interpretation and reviewed the manuscript.

Conflict-of-interest disclosure: E.M. has an advisory mission for Servier. M.B. has performed contract research for Affimed, Amgen, and Regeneron, has served on the Advisory Board for Amgen and Incyte, and has been a member of the Speaker's Bureau for Janssen, Pfizer, and Roche. The remaining authors declare no competing financial interests.

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Footnote

For original data, please contact the corresponding author.

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DOI 10.1182/blood.2020005613

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