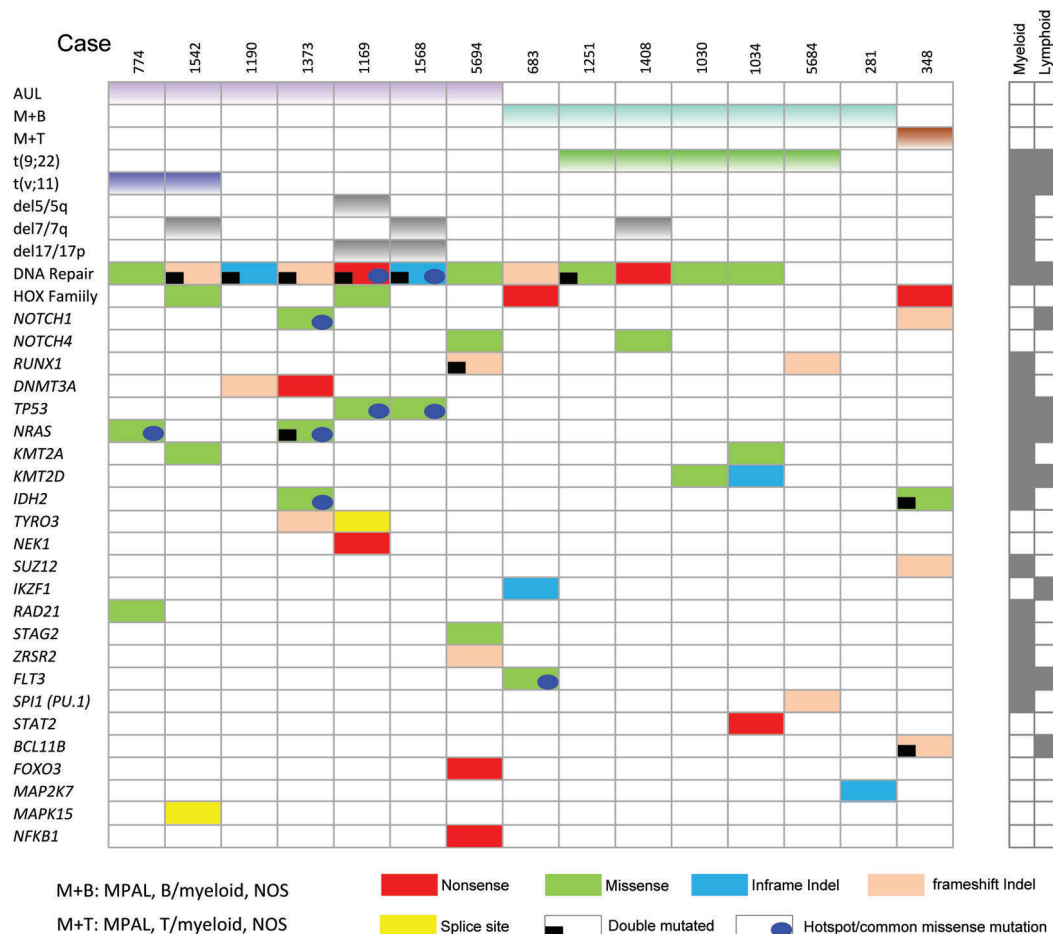


### Mutational and transcriptomic profiling of acute leukemia of ambiguous lineage reveals obscure but clinically important lineage bias

Acute leukemia of ambiguous lineage (ALAL) is a rare group of blood cancers that cannot be clearly classified into either myeloid or lymphoid lineage through traditional immunophenotyping.<sup>1-5</sup> Despite recent prominent leaps in our understanding of the molecular basis of most blood malignancies, ALAL remains a poorly understood leukemic entity, due to its rarity. According to the 2016 update of the World Health Organization classification,<sup>6</sup> ALAL is categorized into five entities: acute undifferentiated leukemia (AUL), mixed phenotype acute leukemia (MPAL) with t(9;22)(q34.1;q11.2) (Philadelphia-chromosome/*BCR-ABL*), MPAL with t(v;11q23.3) (*KMT2A/MLL* rearrangement, v, variable chromosome), MPAL, B/myeloid, not-otherwise-specified (NOS), and MPAL, T/myeloid, NOS. Optimal therapy for ALAL currently remains unclear and patients with ALAL have relatively poorer outcomes compared to patients with acute myeloid or lymphoblastic leukemia.<sup>7,8</sup> A lack of mutational information regarding ALAL raises the question of whether the current classification truly reflects the line-

age underlying the pathogenesis of this group of diseases.<sup>9</sup>

To understand the mutational profile of ALAL and its cellular origins better, we performed whole exome sequencing and transcriptome sequencing on 14 diagnostic samples (diagnosis only) and one case with diagnosis, remission and relapse matched samples (the remission sample was used as a germline control) of ALAL. Our cohort of patients includes seven cases of AUL [2 with t(v;11q23.3)], 5 MPAL with t(9;22)(q34.1;q11.2), 2 MPAL, B/myeloid, NOS, *KMT2A/MLL* rearranged and 1 case of MPAL, T/myeloid, NOS]. (Online Supplementary Table S1). Whole exome sequencing was performed in all 15 cases (>100x, PE150, HiSeq-X10, Illumina) while transcriptome sequencing (PE100, HiSeq-4000, Illumina) was performed on eight samples for which there were adequate cells available for RNA extraction [3 AUL, 3 MPAL with t(9;22)(q34.1;q11.2), and 2 MPAL, B/myeloid, NOS]. Sequencing reads were aligned to the human genome hg19 using BWA and mutations were called using Mutect2. Results were filtered with dbSNP131 – the latest versions were not utilized as they contain some well-characterized somatic oncogenic mutations [e.g., *NRAS* G12D (rs121913237), *IDH2* R140Q (rs121913502)],<sup>10</sup> – 1,000 genome, ExAC, Esp5400 and



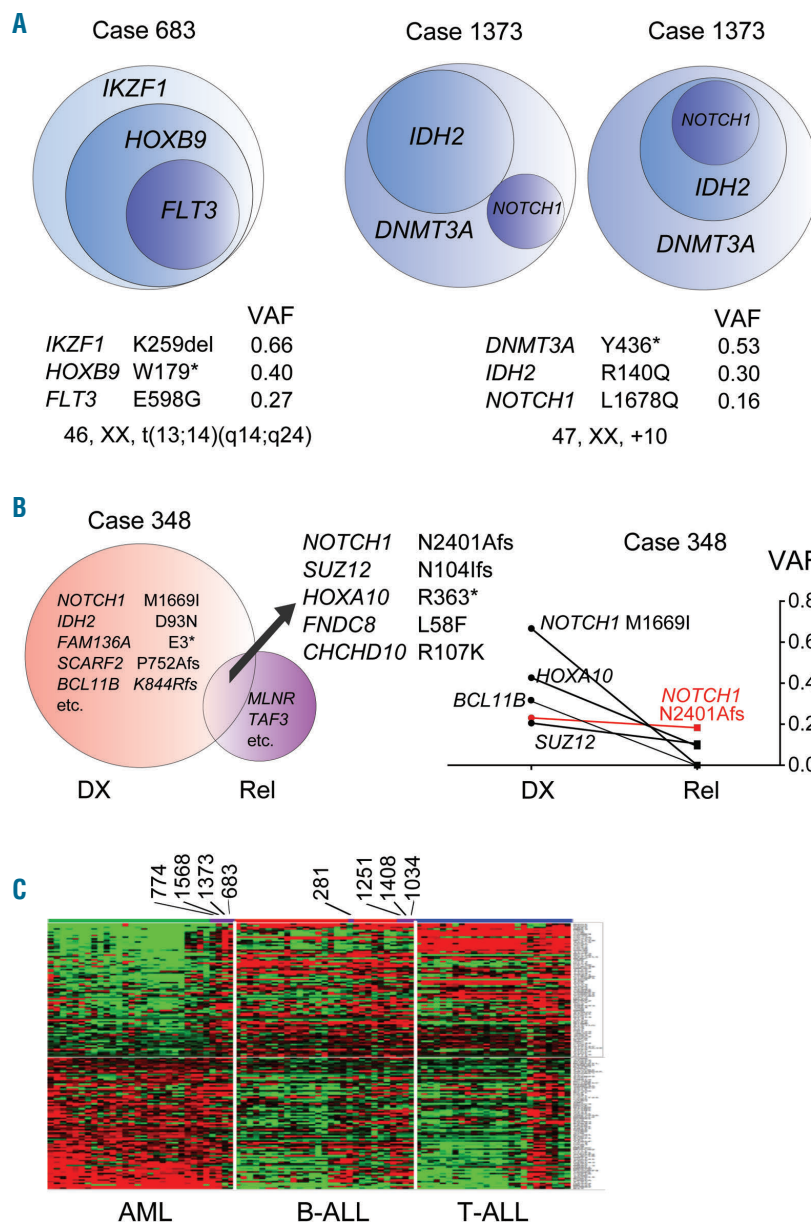
**Figure 1. Mutational heatmap of 15 cases of acute leukemia of ambiguous lineage.** The subtype of acute leukemia of ambiguous lineage (ALAL) – acute undifferentiated leukemia (AUL), mixed phenotype acute leukemia (MPAL), B/myeloid, not otherwise specified (NOS), MPAL, T/myeloid, NOS – and the chromosome abnormality of each sample are indicated. “Double mutated” refers to two mutations that occurred in the same gene in the same sample (in the “DNA repair” row it indicates 2 mutations in genes involved in the same pathway). Gray rectangular boxes on the right indicate genes involved in myeloid or lymphoid neoplasms or both.

an in-house manually curated SNP database.<sup>10,11</sup> Details are provided in the *Online Supplementary Methods*.

Common gene mutations seen in hematopoietic neoplasms were found in most ALAL samples [12 of 15 patients (80%)]. Of note, most mutations were in genes involved in the regulation of either the epigenome or transcription such as *DNMT3A*, *RUNX1*, *TP53* and *KMT2D* (*MLL2*)<sup>10-12</sup> (Figure 1A). Four cases had mutations of either *NOTCH1* or *NOTCH4*. Mutations in *HOX* gene family members occurred in four cases (*HOXA10*, *HOXB2*, *HOXB9* and *HOXD12*). Remarkably, mutations in genes involved in DNA repair pathway occurred in 12 cases (80%) (*Online Supplementary Tables S2 and S3*). While none of these mutations was recurrent, many were frameshift truncations or nonsense mutations, leading to a loss-of-function. For example, one case of MPAL (1408) with t(9;22) harbored a stop-gain mutation of *BRCA2* (S1001\*). A frameshift mutation was found in *PRKDC* (I1085Sfs) in case 683: this gene is involved in the repair of DNA double strand breaks and is required for VDJ

recombination. Homozygous mutation of this gene severely impairs lymphocyte maturation and has been used to generate mice with severe combined immunodeficiency. In addition, a frameshift deletion was found in a Fanconi anemia gene *FANCD2* (Y103Lfs\*77) in case 1542. Mutations also occurred in the DNA damage checkpoint gene *MDC1* (an inframe deletion of 40 amino acids), *CHEK1* and the DNA repair gene *PARP1* (*Online Supplementary Table S2 and S3*). Deleterious common missense mutations of *TP53* occurred in two cases with complex karyotype (1568 and 1169, both also harboring deletion of chromosome 17/17p) (*Online Supplementary Table S1*). The presence of DNA repair pathway mutations in these samples may explain the dysregulation of cell differentiation and poor response to conventional chemotherapy. On the flip side of the coin, deficiencies in DNA repair pathways may be exploited therapeutically to enhance tumor cell killing (e.g., PARP inhibitors in *BRCA* mutated tumors).

Based on the sequencing data, the mixed lineage phe-



**Figure 2. Clonal dynamics and clonal hierarchical model of acute leukemia of ambiguous lineage.** (A) A schematic diagram showing the potential clonal hierarchical model of case 683 (left panel) and case 1373 (right panel). Clonal hierarchical models were inferred based on variant allele frequency (VAF). \*, stop-gain mutation; del, inframe deletion. (B) Left panel, Venn diagram showing mutations present in both diagnostic and relapse samples in case 348. Right panel: schematic diagram displaying alterations of VAF for different genes during leukemic progression. Two *NOTCH1* mutations were detected at diagnosis. At relapse after chemotherapy and complete clinical remission, the clone carrying the M1669I mutation did not reappear, but the clone carrying the N2401Afs mutation survived and grew out. \*, stop-gain mutation; fs, frame shift indel. (C) Cluster analysis of RNA sequencing results of eight ALAL samples, clustered together with RNA sequencing data of acute myeloid leukemia (AML) [25 randomly selected cases from The Cancer Genome Atlas (TCGA) AML cohort], pediatric B-acute lymphocytic leukemia (ALL) and T-ALL (25 randomly selected cases of B-ALL and 25 cases of T-ALL from EGAS00001001858). The cluster analysis was performed using 367 myeloid/lymphoid-expressing signature genes. See also the full picture in *Online Supplementary Figure S1*. Dx: diagnostic sample; Rel: relapse sample.

notype may be partially explained by simultaneous mutations of both hallmark genes associated with myeloid or lymphoid leukemia as well as alteration of the myeloid/lymphoid gene expression signature. For example, case 683 (MPAL, B/myeloid) harbored an inframe deletion of *IKZF1* and a nonsense mutation of *HOXB9*, which are well-known leukemia-associated genes frequently mutated or aberrantly expressed in B-cell acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML), respectively. Analysis of the variant allele frequency of the mutations suggests that the *IKZF1* mutation occurred earlier in the founder clone, prior to acquisition of a *HOXB* stop-gain mutation; while a sub-clonal proliferation driver, *FLT3* mutation (E598G in the juxtamembrane region) was gained later during clonal evolution (Figure 2A, left panel). Similarly, case 1373 (AUL) had *DNMT3A* (stop-gain, Y436\*) and *IDH2* (R140Q) mutations (associated with AML) as well as a T-ALL-associated mutation, *NOTCH1* (L1678Q, hotspot mutation). Two potential clonal hierarchical models were proposed for this case: the leukemic founder clone contained a *DNMT3A* loss-of-function mutation, which subsequently acquired *IDH2* and *NOTCH1* mutations. The *NOTCH1* mutation may have evolved either independently or after the *IDH2* clone (Figure 2A, right panel). Unfortunately, we did not have additional cells to perform single cell sequencing to resolve this dilemma. Case 348 (MPAL T/myeloid) also contained mutations associated with both myeloid (*SUZ12*, frameshift-deletion) and lymphoid (*NOTCH1*) neoplasms. Interestingly, in this case, two *NOTCH1* mutations were detected in the diagnostic sample. These two mutations might belong to different leukemic subclones: at relapse after attaining complete clinical remission following chemotherapy, the clone carrying the M1669I mutation disappeared, but the clone carrying the N2401Afs mutation survived and grew out (Figure 2B).

We next asked whether any clonal B-cell/T-cell receptor (*BCR/TCR*) rearrangements occurred in these samples. To investigate this, we examined the transcripts coding for *BCR/TCR* rearrangements in RNA sequencing data of ALAL samples using MiXCR (<https://mixcr.readthedocs.io>). We did not detect clonal rearrangements of *BCR/TCR* in these eight samples, while our same bioinformatic pipeline readily detected clonal rearrangements of *BCR/TCR* in RNA sequencing data of 230 samples of pediatric ALL<sup>13</sup> and more than 500 lymphoid cell lines.<sup>14</sup>

We observed alterations associated with myelodysplastic syndrome and secondary ALL in patients with AUL. Compared with other ALAL subtypes, AUL cases frequently harbor myelodysplastic syndrome-related chromosomal alterations such as del15/5q, del7/7q and del17/17p, as well as mutations in *DNMT3A*, spliceosome (*ZRSR2*, F236Vfs\*6), *RUNX1* (frameshift deletion, T214Pfs\*23), cohesin complex (*STAG2* and *RAD21*) and *TP53* genes. Analysis of the RNA sequencing data revealed that the expression signature of AUL (cases 774, 1568 and 1373) was closer to that of AML, in contrast to the three cases of MPAL with t(9;22)(q34.1;q11.2) (cases 1034, 1251 and 1408) which clustered within B-ALL (Figure 2C, Online Supplementary Figure S4). When compared to other subtypes of ALAL, AUL patients tended to be older and had a much poorer survival (median survival 8.87 months versus not reached) (Online Supplementary Figure S2). Hence, these AUL patients had a clinical profile similar to that of individuals with secondary AML. This observation suggests that many AUL cases, defined by the World Health Organization monograph,<sup>6</sup> may have a myeloid derivation with myelodysplastic syn-

drome/AML-related mutations. We acknowledge that this conclusion may be biased due to the limited number of cases, and this hypothesis requires examination in a larger cohort of patients.

Conversely, the outcomes of our non-AUL ALAL patients appeared to be better than those of other reported ALAL cohorts (median survival not reached in our patients versus a reported 3-year overall survival rate of 45%<sup>1</sup>). The reason for this improvement is probably that many of our patients (5 out of 8) had MPAL with t(9;22)(q34.1;q11.2) and all were treated with chemotherapy and tyrosine kinase inhibitors against BCR-ABL. In addition, due to their younger age (median=36 years), all of them underwent an allogeneic hematopoietic stem cell transplantation. Both of these therapeutic approaches improve outcomes in MPAL.

During the time we were submitting our manuscript, Takahashi and co-workers reported a related study in which they profiled the mutational landscape of mixed phenotype B/myeloid-T/myeloid leukemia.<sup>15</sup> They showed that mixed phenotype leukemia can often be clustered to either an AML-like or ALL-like MPAL based on methylation profiling. Our study complements their data and showed that while B/myeloid samples clustered mostly with B-ALL, undifferentiated leukemia appears to share a mutational and gene expression profile with that of AML. In addition, as most of our cases of B/myeloid MPAL harbored the t(9;22) translocation, the difference in mutational profile and gene expression suggests that MPAL with t(9;22) is biologically distinct from MPAL/NOS. This difference translates clinically into 70% of these MPAL patients enjoying a 5-year survival compared with less than 20% of the cases studied by Takahashi *et al.*<sup>15</sup>

In conclusion, we characterized the mutational landscape of adult ALAL patients and provide novel insights into this rare leukemic entity, which may help to develop better therapeutic strategies and may alter the treatment paradigm for these patients. AUL might be better treated as AML because of their close genetic, cytogenetic and gene expression association with AML, specifically secondary AML. The rest of the subtype classification of MPAL seem to be biologically and clinically consistent as these leukemias do seem to show a mix of myeloid and lymphoid type mutations and gene expression. These findings suggest that the aberrant cell surface protein expression may not truly represent the hematopoietic cell type that is recapitulated, and gene expression profiling may be a better method to classify these entities. These findings have implications for clinical practice, for example, AUL might be better treated with recently approved therapies such as CPX 351, while the different MPAL may respond better to lineage-appropriate therapy based on their gene expression profile.

Zhen-Tang Lao,<sup>1,2</sup> Ling-Wen Ding,<sup>1,2</sup> Omer An,<sup>1</sup> Norimichi Hattori,<sup>3</sup> Qiao-Yang Sun,<sup>1</sup> Kar-Tong Tan,<sup>4</sup> Anand Mayakonda,<sup>1</sup> Wong Gee Chuan,<sup>2</sup> Vikas Madan,<sup>1</sup> De-Chen Lin,<sup>5</sup> Henry Yang<sup>1</sup> and H. Phillip Koeffler<sup>1,5</sup>

<sup>1</sup>Cancer Science Institute of Singapore, National University of Singapore, Singapore; <sup>2</sup>Department of Haematology, Singapore General Hospital, Singapore; <sup>3</sup>Division of Hematology, Department of Medicine, Showa University, Tokyo, Japan; <sup>4</sup>Division of Medical Sciences, Harvard Medical School, Boston, MA, USA and <sup>5</sup>Division of Hematology/Oncology, Cedars-Sinai Medical Center, UCLA School of Medicine, Los Angeles, CA, USA

Funding: this work was funded by the Leukemia Lymphoma Society of America, by the Singapore Ministry of Health's National Medical Research Council (NMRC) under its Singapore Translational Research



(STaR) Investigator Award (NMRC/STaR/0021/2014), Singapore Ministry of Education Academic Research Fund Tier 2 (MOE2013-T2-2-150), the NMRC Centre Grant awarded to National University Cancer Institute of Singapore (NMRC/CG/012/2013), the National Research Foundation Singapore and the Singapore Ministry of Education under its Research Centres of Excellence initiatives, as well as generous donations from the Melamed family and Reuben Yeroushalmi. This research is also supported by the RNA Biology Center at the Cancer Science Institute of Singapore, NUS, as part of funding under the Singapore Ministry of Education's Tier 3 grants, grant number MOE2014-T3-1-006.

Correspondence: LING-WEN DING.  
dinglingwen@hotmail.com/csdlw@nus.edu.sg  
doi:10.3324/haematol.2018.202911

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at [www.haematologica.org](http://www.haematologica.org).

## References

1. Matutes E, Pickl WF, Van't Veer M, et al. Mixed-phenotype acute leukemia: clinical and laboratory features and outcome in 100 patients defined according to the WHO 2008 classification. *Blood*. 2011;117(11):3163-3171.
2. Wolach O, Stone RM. How I treat mixed-phenotype acute leukemia. *Blood*. 2015;125(16):2477-2485.
3. Gutierrez A, Kentsis A. Acute myeloid/T-lymphoblastic leukaemia (AMTL): a distinct category of acute leukaemias with common pathogenesis in need of improved therapy. *Br J Haematol*. 2018;180(6):919-924.
4. Eckstein OS, Wang L, Punia JN, et al. Mixed-phenotype acute leukemia (MPAL) exhibits frequent mutations in DNMT3A and activated signaling genes. *Exp Hematol*. 2016;44(8):740-744.
5. Porwit A, Bene MC. Acute leukemias of ambiguous origin. *Am J Clin Pathol*. 2015;144(3):361-376.
6. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127(20):2391-2405.
7. Muntean AG, Hess JL. The pathogenesis of mixed-lineage leukemia. *Annu Rev Pathol*. 2012;7:283-301.
8. Slany RK. The molecular mechanics of mixed lineage leukemia. *Oncogene*. 2016;35(40):5215-5223.
9. Weinberg OK, Arber DA. Mixed-phenotype acute leukemia: historical overview and a new definition. *Leukemia*. 2010;24(11):1844-1851.
10. Sun QY, Ding LW, Tan KT, et al. Ordering of mutations in acute myeloid leukemia with partial tandem duplication of MLL (MLL-PTD). *Leukemia*. 2017;31(1):1-10.
11. Ding LW, Sun QY, Tan KT, et al. Mutational landscape of pediatric acute lymphoblastic leukemia. *Cancer Res*. 2017;77(2):390-400.
12. Ding L, Ley TJ, Larson DE, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature*. 2012;481(7382):506-510.
13. Ding LW, Tan KT, Sun QY, et al. Clonality and clonal evolution analysis of paediatric ALL based on B-cell receptor/T-cell receptor rearrangement. *Br J Haematol*. 2019;184(5):829-833.
14. Tan KT, Ding LW, Sun QY, et al. Profiling the B/T cell receptor repertoire of lymphocyte derived cell lines. *BMC Cancer*. 2018;18(1):940.
15. Takahashi K, Wang F, Morita K, et al. Integrative genomic analysis of adult mixed phenotype acute leukemia delineates lineage associated molecular subtypes. *Nat Commun*. 2018;9(1):2670.