Exclusion of persistent mutations in splicing factor genes and isocitrate dehydrogenase 2 improves the prognostic power of molecular measurable residual disease assessment in acute myeloid leukemia

Accurate risk assessment is crucial for the management of patients with acute myeloid leukemia (AML).[1] The detection of measurable residual disease (MRD) after remission induction therapies has been shown to be an independent risk factor for relapse and death.[2] The use of next-generation sequencing (NGS)-based techniques to detect mutations found in leukemic cells has emerged as a promising approach for MRD assessment.[3,4] One of the main challenges of this approach is differentiating between mutations that are found only in the leukemic cell population (henceforth termed "AML-related") and those associated with clonal hematopoiesis (CH). The persistence of CH during remission has not been associated with inferior clinical outcomes.[5] Approaches involving genotyping of sorted populations or single cells are required to identify the cellular origins of the mutations, but they are not yet practical for routine clinical use.

To overcome this challenge, a common practice is to exclude mutations in three genes, namely *DNMT3A*, *TET2*, and *ASXL1* (collectively known as DTA), from molecular MRD assessment,[1,3,4] because they are among the most frequently mutated genes in people with clonal hematopoiesis of indeterminate potential (CHIP).[6,7] However, mutations in other genes are also found in CHIP carriers.[6-8] Moreover, the relative frequencies of CH-related mutations in AML patients differ from those of CHIP carriers who, by definition, do not have any other hematologic abnormalities.[5] This discordance is likely because the risk of AML development varies between different CH-related mutations.[8] Thus, it is unclear whether DTA mutations are the optimal ones for exclusion in molecular MRD analysis in AML.

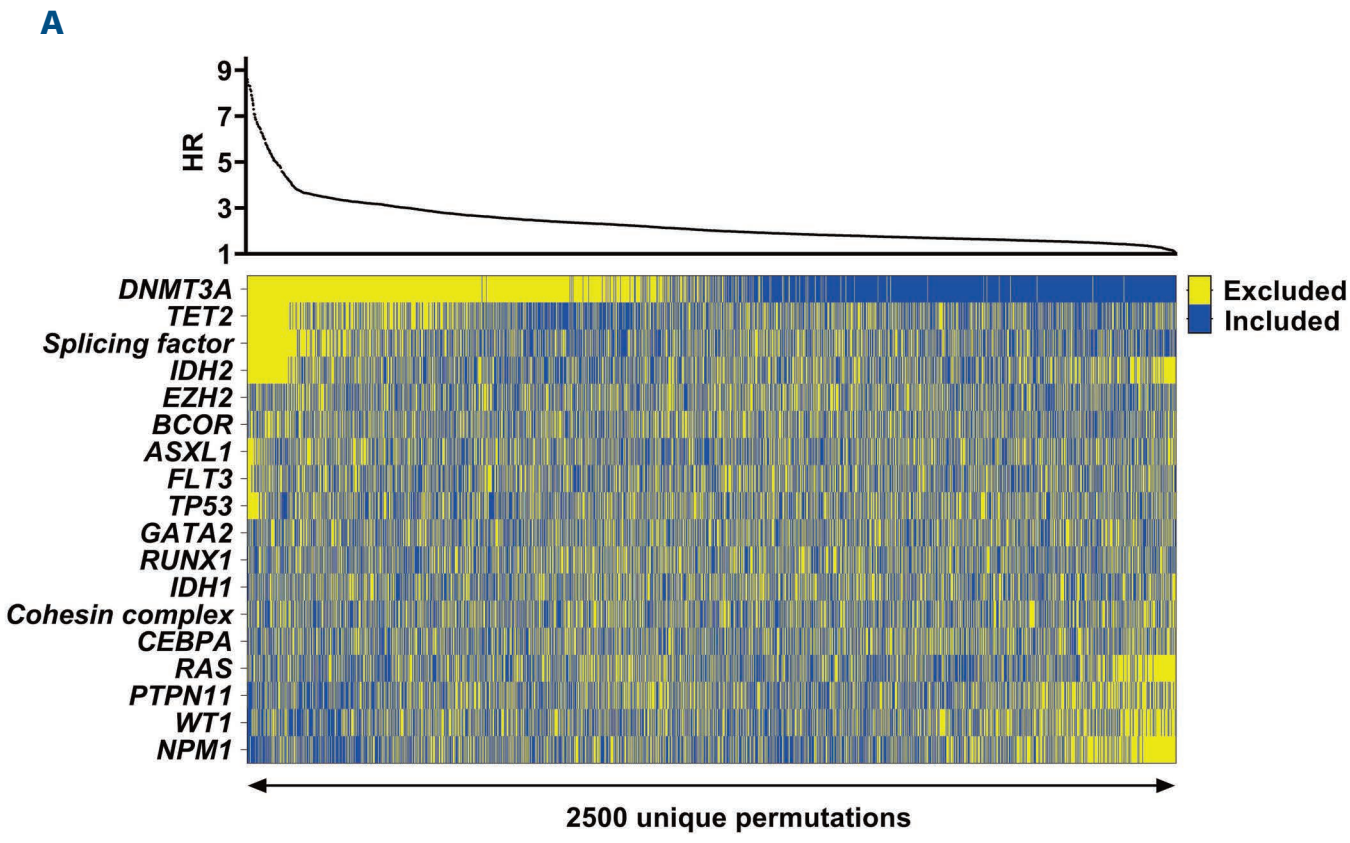
To address the above uncertainty, we systematically analyzed the impact of exclusion of mutations in 22 myeloid malignancy-associated genes on the difference in clinical outcomes between patients stratified as MRD-positive (MRD^{POS}) and MRD-negative (MRD^{NEG}). To perform this analysis, we studied 114 newly diagnosed AML patients who received high-intensity induction chemotherapy and achieved a complete remission. The clinical characteristics of the patients are listed in *Online Supplementary Table S1*. We performed targeted conventional NGS analysis on DNA extracted from their diagnostic peripheral blood or bone marrow samples. Variants classified as "benign" or "likely benign" based on American College of Medical Genetics criteria were excluded from further analysis.[9] During the remission phase, we collected a total of 223 peripheral blood samples upon count recovery at a median of 36 days after induction (n=93) or after one (n=93), two (n=35), or three (n=2) cycles of consolidation chemotherapy. Remission samples were collected at two different timepoints (designated as T1 and T2) for 95.6% (n=109) of the patients. The remaining patients (n=5) had one remission sample collected at T1. To detect mutations in the remission samples, we used a custom 37-gene hybrid capture panel and error-corrected NGS based on the duplex sequencing approach.[10,11]

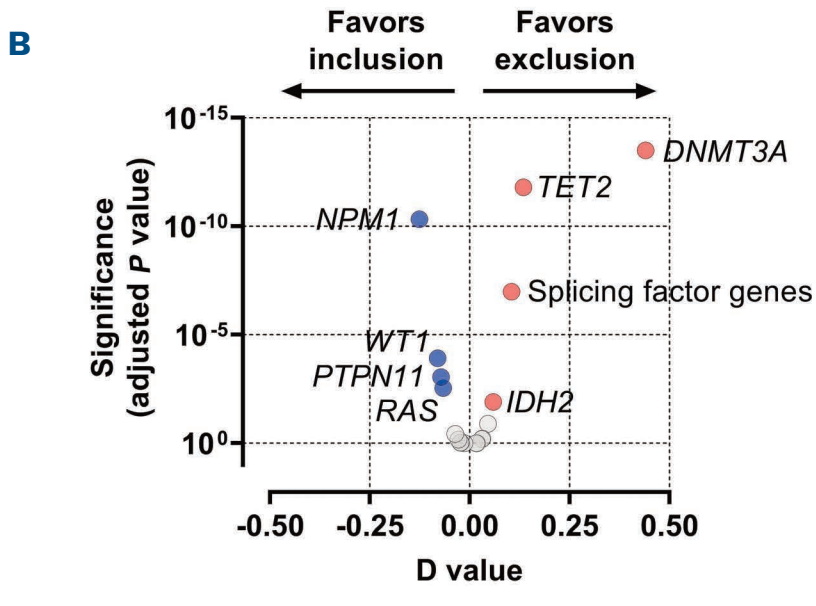
A total of 336 mutations in 35 genes were identified and passed the American College of Medical Genetics filtering step in the diagnostic samples. Of those, we excluded 26 mutations in genes that were not covered by the custom panel for MRD detection from further analysis. A further 13 mutations in five genes (*PHF6*, *KDM6A*, *JAK2*, *KIT*, *CBL*) were excluded because of a low number of mutational events per gene (≤4 events). The remaining 297 mutations were distributed across 22 genes in 101 patients. Mutations in genes that share a common pathogenic mechanism were analyzed as a group. The groups were splicing factor mutations (*SRSF2*, *SF3B1*, and *U2AF1*), RAS mutations (*KRAS* and *NRAS*), and cohesin complex mutations (*RAD21* and *STAG2*). Mutations in *IDH1* and *IDH2* were analyzed separately because of recent evidence demonstrating distinct clinical and co-mutational patterns between the two types of mutations in patients with myeloid malignancies.[12]

The use of duplex sequencing enabled sensitive and accurate measurement of the allele frequency of each mutation in the remission samples. The distribution of mutant allele frequencies at T1 (*Online Supplementary Figure S1A*) and stability of the mutations between T1 and T2 were highly variable across the genes (*Online Supplementary Figure S1B*). At one end of the spectrum were mutations that demonstrated high levels of persistence and stability during remission, such as *DNMT3A* mutations. At the other end were mutations characterized by lower levels of persistence and higher probability of clearance with chemotherapy, such as *NPM1* mutations. The characteristics of most mutations fell somewhere between

the two extremes. It is noteworthy that some mutations, including mutations in the splicing factor genes and *IDH2*, demonstrated a comparable level of persistence and stability as *DNMT3A* mutations.

To evaluate the impact of mutations in each gene on MRD analysis in an unbiased manner, we generated 2,500 unique permutations in which each of the 15 genes or three gene groups was randomly assigned to be included or excluded from MRD assessment. For each permutation, we calculated the hazard ratio for overall survival between MRD^{POS} and MRD^{NEG} patients in our study cohort. Patients with mutations in any of the included genes above a mutant allele frequency cutoff of 0.01 (1%) at T1 or T2 were considered MRD^{POS}. Permutations that excluded *DNMT3A* and *TET2* mutations were associated with higher hazard ratios, whereas permutations that excluded well-characterized AML-related mutations, such as *NPM1* and RAS mutations, correlated with lower hazard ratios (Figure 1A). To determine the significance and magnitude of these associations, we used the Kolmogorov-Smirnov test to compare the distribution of hazard ratios among the subset of permutations in which a specific gene or gene group was excluded with the reference distribution of all 2,500 permutations. This analysis showed that exclusion of *DNMT3A* or *TET2* mutations significantly shifted the hazard ratio distribution higher relative to the reference distribution (Figure 1B). Intriguingly, exclusion of mutations in the splicing factor genes or *IDH2* also significantly shifted the hazard ratio distribution higher (Figure 1B), and their inclusion eliminated the highest hazard





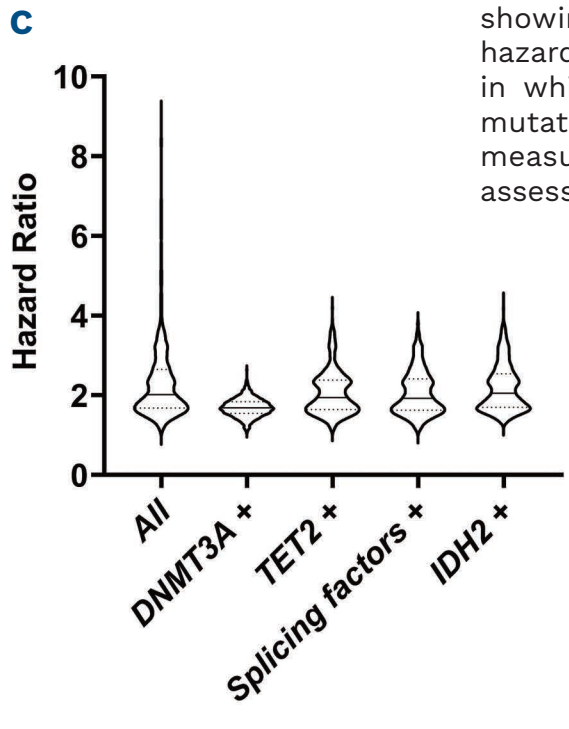


Figure 1. Evaluation of the impact of exclusion or inclusion of myeloid malignancy-associated mutations on the prognostic power of molecular measurable residual disease assessment. (A) Heatmap showing each of the 2,500 unique permutations ordered according to their associated hazard ratios for overall survival. A yellow cell indicates exclusion of the indicated gene mutation for measurable residual disease assessment, whereas a blue cell indicates inclusion. (B) Volcano plot showing statistical significance plotted against the D statistic from the Kolmogorov-Smirnov test comparing the distribution of hazard ratios of permutations in which the indicated gene mutation is excluded *versus* the reference distribution. See text for details. (C) Violin plots showing the distribution of hazard ratios of permutations in which the indicated gene mutation is included (+) for measurable residual disease assessment. HR: hazard ratio.

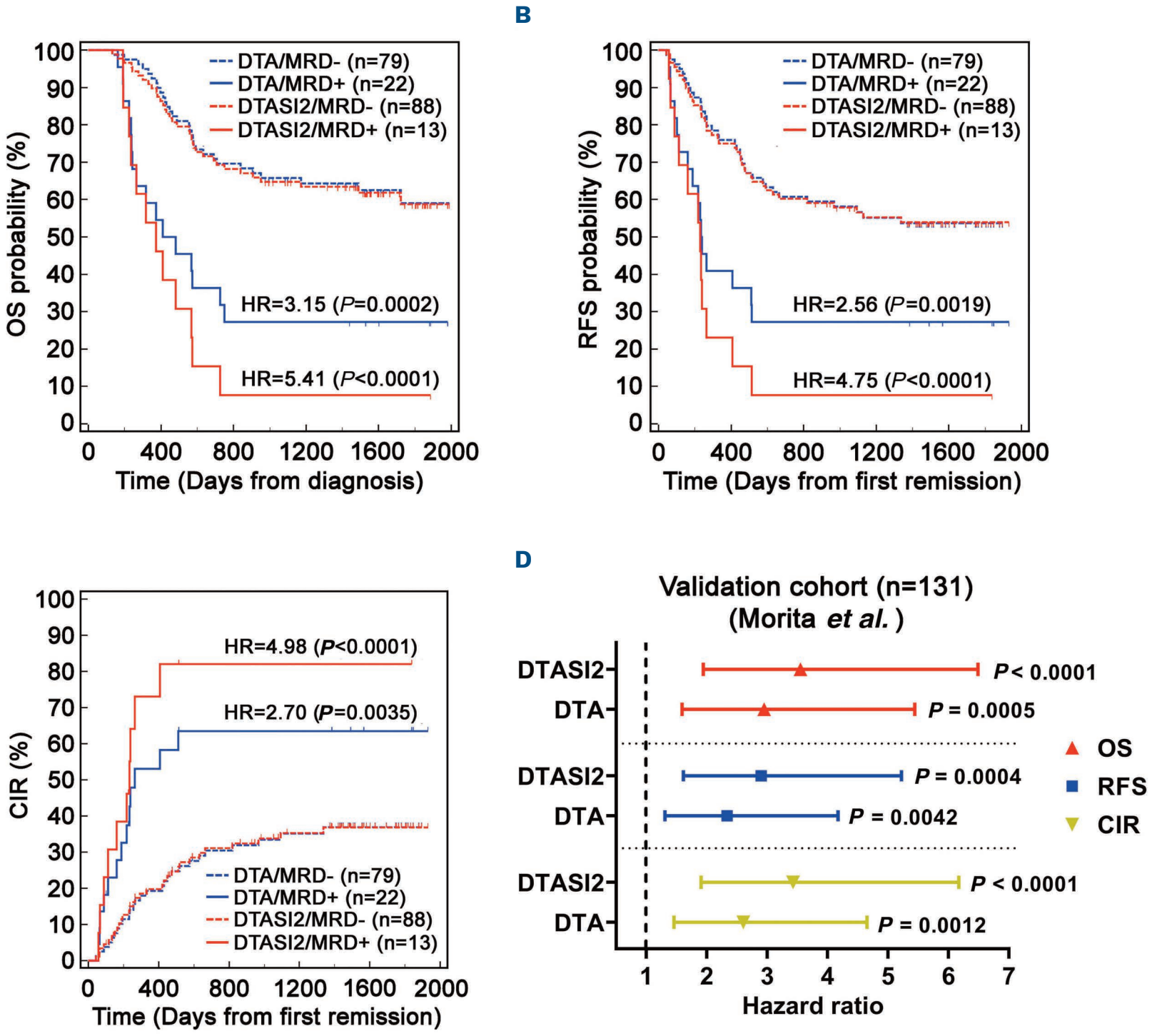


Figure 2. Exclusion of mutations in splicing factor genes and *IDH2* in addition to *DNMT3A*, *TET2*, and *ASXL1* mutations improves the prognostic power of molecular measurable residual disease assessment. Kaplan-Meier plots for (A) overall survival (OS), (B) relapse-free survival (RFS), and (C) cumulative incidence of relapse (CIR) of patients classified as measurable residual disease (MRD)-positive (MRD+) or MRD-negative (MRD-) and based on whether *DNMT3A*, *TET2*, and *ASXL1* (DTA) mutations or mutations in DTA, splicing factor genes and *IDH2* (DTAS12) were excluded from MRD determination. A mutant allele frequency of >0.01 (1%) at timepoints 1 and 2 was used to define MRD positivity. Comparison of the hazard ratios for OS, RFS, and CIR in the Morita *et al.* (D) or Ahn *et al.* (E) validation cohort between exclusion of mutations in DTA alone *versus* DTAS12 for determination of MRD status. A mutant allele frequency of >0.01 (1%) was used to define MRD positivity. The hazard ratios and *P* values shown in all panels were calculated using the Cox proportional-hazards model.

ratio values found in the reference distribution (Figure 1C). These findings, along with their high level of persistence and stability during remission, suggest that mutations in the splicing factor genes and *IDH2* should be considered CH-related for the purpose of molecular MRD assessment. Indeed, exclusion of these mutations in addition to DTA (henceforth referred to as "DTASI2") led to a greater difference in overall survival, relapse-free survival, and cumulative incidence of relapse between MRD^{POS} and MRD^{NEG} patients using a mutant allele frequency cutoff at 0.01 (1%) or 0.005 (0.5%) when compared with exclusion of DTA mutations alone or no mutations (Figure 2A-C, *Online Supplementary Figure S2A-C*). To ensure that these observations were not restricted to our cohort of patients and the analysis of peripheral blood samples, we analyzed two independent datasets that used panel-based targeted NGS for MRD monitoring of bone marrow samples in AML patients in remission.[4,13] The total numbers of patients in the cohorts studied by Morita *et al.*[4] and Ahn *et al.*[13] were 131 and 124, respectively. In both validation cohorts, exclusion of DTASI2 mutations increased the hazard ratio for overall survival, relapse-free survival, and cumulative incidence of relapse in MRD^{POS} patients compared with exclusion of DTA mutations alone (Figure 2D, E).

Our findings demonstrate that the exclusion of mutations in splicing factor genes and *IDH2* improves the prognostic power of NGS-based MRD assessment. This effect is likely attributable to their involvement in CH. Our results are consistent with those of a recent study showing that the persistence of *IDH1/2* and *SRSF2* mutations had no impact on survival in *NPM1*-mutated AML patients.[14] Due to the size of our study cohort, the potential impact of mutations in other genes with low representation could have been missed. Notably, our analysis did not identify *ASXL1* mutations as CH-related. However, the number of patients with *ASXL1* mutations in our cohort was small (n=9). Further studies are required to clarify the significance of persistence of *ASXL1* mutations in MRD analysis. In addition, it is important to emphasize that patients in our study cohort were treated with intensive chemotherapy and thus our findings may not be applicable to patients treated with other therapies (e.g., venetoclax-based regimens). Our work highlights the importance of optimizing the definition of CH-related gene mutations for molecular MRD assessment.

Authors

Tracy Murphy,[1*] Jinfeng Zou,[1*] Andrea Arruda,[1] Ting Ting Wang,[1] Zhen Zhao,[1] Yangqiao Zheng,[1] Vikas Gupta,[1] Dawn Maze,[1] Caroline D. McNamara,[2] Mark D. Minden,[1] Aaron D. Schimmer,[1] Hassan Sibai,[1] Karen W. Y. Yee,[1] Jose-Mario Capo-Chichi,[1,3] Tracy L. Stockley,[1,3] Andre C. Schuh,[1] Scott V. Bratman[1#] and Steven M. Chan[1#]

[1]Princess Margaret Cancer Center, University Health Network, Toronto, Ontario, Canada; [2]Royal Brisbane and Women's Hospital, Herston, Queensland, Australia and [3]Laboratory Medicine Program, University Health Network, Toronto, Ontario, Canada

TM and JZ contributed equally as first authors.

#SVB and SMC contributed equally as senior authors.

Correspondence:
STEVEN M. CHAN - steven.chan@uhnresearch.ca

https://doi.org/10.3324/haematol.2023.283510

Received: May 10, 2023.
Accepted: June 14, 2023.
Early view: June 22, 2023.

Disclosures

No conflicts of interest to disclose.

Contributions

TM, JZ, SVB, and SMC designed and performed the research, analyzed the data, and wrote the paper. VG, DM, CJM, MDM, ADS, HS, KWLY, and ACS managed and treated the patients. AA, TTW, ZZ, YZ, J-MC-C, and TLS performed the research.

Acknowledgments

The authors thank the leukemia tissue bank and the Liquid Biopsy Evaluation and Repository Development (LIBERATE) study team at Princess Margaret Cancer Centre for the collection and storage of samples. The authors also thank Dr. Dennis Kim at Princess Margaret Cancer Centre and Dr. Koichi Takashashi at MD Anderson Cancer Center for providing clinical data of the independent cohorts.

Funding

This work was supported by a Collaborative Personalized Cancer Medicine Team Grant from the Princess Margaret Cancer Foundation and an Acute Leukemia Translational Research Initiative Grant from the Ontario Institute for Cancer Research.

Data-sharing statement

Sequencing and mutation data are available upon request to the corresponding author.

References

1. Dohner H, Wei AH, Appelbaum FR, et al. Diagnosis and management of AML in adults: 2022 ELN recommendations from an international expert panel. Blood. 2022;129(4):424-447.
2. Walter RB, Ofran Y, Wierzbowska A, et al. Measurable residual disease as a biomarker in acute myeloid leukemia: theoretical and practical considerations. Leukemia. 2021;35(6):1529-1538.
3. Jongen-Lavrencic M, Grob T, Hanekamp D, et al. Molecular minimal residual disease in acute myeloid leukemia. N Engl J Med. 2018;378(13):1189-1199.
4. Morita K, Kantarjian HM, Wang F, et al. Clearance of somatic mutations at remission and the risk of relapse in acute myeloid leukemia. J Clin Oncol. 2018;36(18):1788-1797.
5. Tanaka T, Morita K, Loghavi S, et al. Clonal dynamics and clinical implications of postremission clonal hematopoiesis in acute myeloid leukemia. Blood. 2021;138(18):1733-1739.
6. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. N Engl J Med. 2014;371(26):2488-2498.
7. Genovese G, Kahler AK, Handsaker RE, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. N Engl J Med. 2014;371(26):2477-2487.
8. Fabre MA, de Almeida JG, Fiorillo E, et al. The longitudinal dynamics and natural history of clonal haematopoiesis. Nature. 2022;606(7913):335-342.
9. Kopanos C, Tsiolkas V, Kouris A, et al. VarSome: the human genomic variant search engine. Bioinformatics. 2019;35(11):1978-1980.
10. Kennedy SR, Schmitt MW, Fox EJ, et al. Detecting ultralow-frequency mutations by duplex sequencing. Nat Protoc. 2014;9(11):2586-2606.
11. Murphy T, Zou J, Daher-Reyes GS, et al. Impact of preleukemic mutations and their persistence on hematologic recovery after induction chemotherapy for AML. Blood Adv. 2019;3(15):2307-2311.
12. Middeke JM, Metzeler KH, Rollig C, et al. Differential impact of IDH1/2 mutational subclasses on outcome in adult AML: results from a large multicenter study. Blood Adv. 2022;6(5):1394-1405.
13. Ahn JS, Kim T, Jung SH, et al. Allogeneic transplant can abrogate the risk of relapse in the patients of first remission acute myeloid leukemia with detectable measurable residual disease by next-generation sequencing. Bone Marrow Transplant. 2021;56(5):1159-1170.
14. Cappelli LV, Meggendorfer M, Baer C, et al. Indeterminate and oncogenic potential: CHIP vs CHOP mutations in AML with NPM1 alteration. Leukemia. 2022;36(2):394-402.