Epigenetic modifier gene mutations in chronic myeloid leukemia (CML) at diagnosis are associated with risk of relapse upon treatment discontinuation

TO THE EDITOR:

The implementation of tyrosine kinase inhibitors (TKIs) has dramatically improved the outcome of CML patients with the overall survival approaching general population [1]. Hence, the achievement of durable treatment-free remission (TFR) after TKI discontinuation has emerged as a new treatment goal [2]. In several studies, approximately half of CML patients in deep molecular remission (DMR) can successfully maintain TFR after TKI discontinuation [3]. Factors such as the duration of TKI treatment and DMR prior to TKI stop, the 3 month halving time, as well as the number and function of immune cells, namely NK cells, have been suggested to affect and predict the outcome of TKI discontinuation [4–6].

Several recent studies have reported the association of somatic mutations involving cancer-associated genes with treatment outcome in chronic phase (CP) CML [7–10]. Mutations in epigenetic modifier genes, such as ASXL1 and DNMT3A, represent the major fraction of mutations in CP-CML [11]. Despite the epigenetic modifier gene mutations being reported to associate with inferior responses to TKI therapy [12], such mutations can also be found in patients achieving DMR [7, 8] who would be eligible for attempting TKI discontinuation. The role of somatic mutations in predicting TFR has not been explored.

To investigate the potential effect of epigenetic modifier gene variants on the outcome of TKI discontinuation, we analyzed diagnostic samples from 47 CML patients who have attempted TKI discontinuation using targeted sequencing of selected cancer-associated genes. Our cohorts included 32 patients from the Helsinki University Hospital (cohort 1) and 15 patients monitored in South Australia (cohort 2). Only the availability of sample from the time of diagnosis and later TKI discontinuation attempt were used as selection criteria for the study. Loss of major molecular response was encountered in 30 of 47 (64%) patients after TKI discontinuation, while 17 of 47 (36%) patients maintained TFR. The median follow-up time of patients who maintained TFR is 71 months (12–138 months). Diagnostic samples were selected for mutation profiling to enable the identification of somatic cancer-associated mutations in CML leukemic cells, as at the time of TKI discontinuation after sustained DMR, patients typically have undetectable amount of leukemia cells left. Details on patient characteristics and experimental methods are included in the Supplemental Tables 1, 2 and Supplemental materials.

Overall, we identified cancer-associated mutations in 12 of 47 patients (26%) (Table 1), consistent with previous studies reporting the presence of mutations to be 20% in CP patients with optimal response to TKIs [7–10]. The majority of variants was identified in diagnostic samples from patients who relapsed after TKI discontinuation (10/30 patients, 33%) compared to patients maintaining TFR (2/17 patients, 12%, p = 0.052) (Fig. 1a and Supplemental Fig. 1). Mutations in the epigenetic modifier genes were the most common mutation type (9/12, 75%), ASXL1 was the most frequently mutated gene in 5 patients (Table 1). Other mutated epigenetic modifier genes included KDM6A and DNMT3A. Overall, mutations in epigenetic modifier genes were more frequently encountered in patients who relapsed after TKI discontinuation (8/30 patients, 27%) compared to patients who maintained TFR (1/17 patients, 6%), p = 0.041 (Fig. 1b and Supplemental Fig. 1).

Univariable and multivariable logistic regression analyses were performed for predictive factors of TFR (variables used in the analyses are listed in the Supplemental Table 3). Immunological parameters were also included from the Euro-Ski patients [5] in cohort 1. The presence of an epigenetic modifier gene variant and the duration of TKI treatment prior to stop were predictive of the TKI stop outcome in the univariable analysis (Supplemental Table 3). Patients with mutations in epigenetic modifier genes had worse relapse-free survival (RFS) rates compared to patients without mutations (median RFS 3.2 and 16.5 months respectively, p = 0.024, hazard ratio = 3.55, 95% CI: 1.18–10.69) (Fig. 1c). We also performed multivariable analysis, but only borderline significant values were observed for the on-TKI duration and presence of mutations at diagnosis (Supplemental Table 3). Integration of the on-TKI duration to the epigenetic mutation slightly improved the separation of groups, but the presence of the epigenetic mutations seemed to be the strongest predictor of relapse after TKI discontinuation (Fig. 1d).

ASXL1 was the only recurrently mutated gene in our study, and it was detected in one patient who maintained TFR and four relapse patients. This is in agreement with the reported controversial prognostic value of ASXL1 mutations in CP-CML [12]. Variants in other epigenetic modifier genes, such as KDM6A, were only identified in relapse patients. Recurrent KDM6A mutations have been previously reported in CP-CML [7]. KDM6A is a histone lysine demethylase and a tumor suppressor, that is recurrently mutated in AML (acute myeloid leukemia) [13], multiple myeloma (MM) [14], and solid tumors [15]. KDM6A is a key regulator of the development and the phenotype of various immune cells, such as NK [16], NKT cells [17], and T cells [18]. KDM6A has been reported to modulate immune surveillance in MM, via the control of expression of major histocompatibility complex I and II molecules [19]. Furthermore, KDM6A deletions in medulloblastoma have been shown to impair immune cells recruitment [15].
### Table 1. Cancer-related gene mutations in CML patients attempting TKI discontinuation.

| Patient | Outcome | Variant | Gene | Predicted effect | AA Change | VAF |
|---------|---------|---------|------|-----------------|-----------|-----|
| pt_1    | relapse | 20-32434638-A-G | ASXL1<sup>a</sup> | frameshift deletion | p.G643fs | 5%  |
| pt_2    | relapse | X-45079223-C-T | KDM6A<sup>a</sup> | nonsense | p.Q1058X | 6%  |
| pt_4    | relapse | 7-152177073-G-A | KMT2C<sup>a</sup> | missense | p.P2794S | 44% |
| pt_5    | relapse | 12-49024702-C-T | KMT2D<sup>a</sup> | missense | p.G5310R | 27% |
| pt_9    | relapse | 1-85270827-AT-A | BCL10 | frameshift deletion | p.I46fs | 7%  |
| pt_12   | relapse | 9-77922307-T-G | GNAQ | missense | p.M59L | 6%  |
| pt_33   | relapse | 20-32435461-G-T | ASXL1<sup>a</sup> | nonsense | p.E917<sup>a</sup> | 50% |
| pt_34   | relapse | 20-32434789-C-T | ASXL1<sup>a</sup> | nonsense | p.R693<sup>a</sup> | 39% |
| pt_35   | relapse | 20-32436404-CCA-A | ASXL1<sup>a</sup> | frameshift deletion | p.S1231fs | 7%  |
| pt_36   | relapse | 2-25243930-G-A | DNMT3A<sup>a</sup> | missense | p.R635Q | 43% |
| pt_22   | TFR     | 20-32434638-A-G | ASXL1<sup>a</sup> | frameshift insertion | p.G642fs | 31% |
| pt_47   | TFR     | 2-34880643-C-A | RUNX1 | nonsense | p.S141* | 17% |

Outcome: outcome of TKI discontinuation, AA amino acid, VAF variant allele frequency. *Indicates epigenetic modifier genes.

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**Fig. 1** The impact of epigenetic modifier gene mutations on the outcome of TKI discontinuation. Stacked columns comparing the prevalence of mutations in a cancer-associated genes and b epigenetic modifier genes between relapse and TFR patient groups (n = 47, 30 relapse and 17 TFR patients). Cancer-associated mutations were identified in 10/30 relapse patients compared to 2/17 TFR patients. Epigenetic modifier gene mutations were identified in 8/30 relapse patient compared to 1/17 TFR patients. The list of the identified mutations is provided in Table 1. Survival curves comparing the relapse-free survival rates of patients (n = 47) classified according to c the presence of epigenetic modifier gene mutations using log-rank test, d combinations of epigenetic modifier mutations and duration of TKI treatment prior to stop using stratified log-rank test. Scatter plots showing e the sensitivity of K562-KDM6A-KO cells to NK cells (expanded from freshly isolated NK cells from two different healthy donors’ buffy coats) induced cytotoxicity compared to control K562 cells, f Expression of CD107a degranulation marker on CD56<sup>+</sup> NK cells cocultured with either K562_control or K562_KDM6A_KO cells (clones #1 and #2). (*) p < 0.05, Chi-square test.
Given the suggested important role of immune responses in TFR and the reported role of KDM6A in modulating tumor immune responses, we next investigated the functional consequences of KDM6A mutations in CML cells as an example of relapse-associated epigenetic modifier mutation. At first, we sorted granulocyte, T-, NK-, and NK-T-cell populations from diagnosis and remission samples from the patient who relapsed after TKI discontinuation and had a somatic stop-gain KDM6A mutation. Deep amplicon sequencing was used to detect KDM6A mutation from the sorted fractions, and interestingly, in the diagnostic samples we identified KDM6A mutation to be present in both granulocyte and NK-cell populations at a comparable VAF (8% and 6%, respectively), but not in the T- or NK-T-cell populations. The mutation was not found in any population in the remission samples, suggesting the leukemic origin of the mutation, and that KDM6A-mutated NK cells at diagnosis were part of the malignant clone. This was confirmed by the detection of BCR-ABL hybrid in NK cells at diagnosis (Supplemental Fig. 3a). Next, we wanted to investigate the potential effect of KDM6A mutations on the immune interactions between CML and NK cells. Using CRISPR/Cas9 gene editing, we introduced knockout (KO) of KDM6A gene in K562 CML cells. While the effects of KDM6A-KO were notable on the status of histone acetylation and methylation in K562 cells, there was no change in the sensitivity of K562 cells to TKIs (Supplemental Fig. 3b, c). Interestingly, K562-KDM6A-KO cells exhibited reduced sensitivity to NK-cell mediated cytotoxicity at different ET ratios, compared to control cells (p < 0.001). The reduced sensitivity of K562-KDM6A-KO cells was preserved using either expanded/activated NK cells or freshly isolated NK cells from healthy donors (Fig. 1e, Supplemental Fig. 3d, e). In accordance, NK cells showed reduced surface expression of CD107a (degranulation marker) when cocultured with K562-KDM6A-KO cells compared to coculture with control cells (Fig. 1f). In contrast, knockout of KDM6A in the cytotoxic NK-cell line, NK-92, was not associated with reduced cytotoxic activity against K562 cells (Supplemental Fig. 3f). Gene expression data from K562-KDM6A-KO cells revealed downregulation of allograft rejection and immune regulatory pathways, while drug transporters and EZH2-targets were among the most upregulated genes (Supplemental Fig. 4). Interestingly, KDM6A loss has been reported to enhance tumorigenicity of MM cells through unopposed EZH2 activity [14]. We also reanalyzed previously published [13] gene expression data of K562 with KDM6A knockdown, and the antigen processing and presentation pathway was similarly among the most downregulated pathways in KDM6A knockdown cells.

In conclusion, our study provides novel insights of the potential impact of somatic mutations detected at diagnosis on the outcomes of treatment discontinuation in CML. The detection of mutations at the diagnosis can potentially contribute to the choice of frontline TKI, to negate the effect of mutations on the clinical outcome. Accordingly, recent studies have shown that the frontline use of second-generation TKIs potentially overcome the negative impact of epigenetic modifier gene mutations on CML CP patients’ treatment response [8]. Our findings also suggest a potential link between some of the detected mutations, e.g., KDM6A mutations, and impaired immune responses in CML. Such mutations occur, however, in a small number of relapsed patients and cannot explain all relapses. Thus, characterization of larger patient groups is needed to increase understanding of the potential role of mutations in modulating CML immune responses. To date, genetic screening is not included in the current CML guidelines, despite recent evidence of somatic mutations contributing to treatment outcomes. Further studies are warranted to investigate the potential predictive value of genetic data as a biomarker for relapse after TKI stop, which would enable better selection of eligible patients for treatment discontinuation.
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ACKNOWLEDGEMENTS
We thank the staff of Hematology Research Unit Helsinki (Saara Vaalas, Minna Pajuportti), and Sequencing Laboratory Unit (Pekka Ellonen, Sari Hannula, Tiina Hannunen) at the Institute for Molecular Medicine Finland (FIMM) for their excellent technical assistance. We thank Karsten Spiekermann and Sabrina Weser for providing gene expression data from KDM6A knockdown experiment in K562 cell line. CSC (IT center for science LTD) is acknowledged for their expert help and computing resources. This work was supported by Academy of Finland, the Finnish Funding Agency for Innovation (Dnro 6113/31/2016), Finnish special governmental subsidy for health sciences, research, and training, Signe and Ane Gyllenberg Foundation, Sigrid Juselius Foundation, Finnish Cultural Foundation, Ida Montin Foundation, K. Albin Johanssons stiftelse Foundation, Magnus Ehrnrooth Foundation, Orion Pharmos Research grant, Nordic Cancer Union, Helsinki Institute of Life Science, Relander Foundation, Incyte Nordic Hematology grant and Cancer Foundation Finland. FIMM sequencing unit is supported by HiLIFE and Biocenter Finland.

AUTHOR CONTRIBUTIONS
S.A.A. designed the study, performed experiments, analyzed data from DNA, RNA-sequencing, and coculture cytotoxicity analyses. O.B. contributed to multivariable analyses. N.S., T.P.H., and S.B. contributed to collection of biological samples and clinical data as well as production and analysis of sequencing data from cohort 2. H.L., J.K., and H.I. contributed to CRISPR-editing and coculture cytotoxicity experiments. T. J. and M.K performed DNA sequencing data analyses. S.K. and P.K. contributed to preparation and collection of biological samples and clinical data. S.M. conceived and designed the study, directed and supervised the research. S.A.A and S.M. wrote the manuscript. All authors contributed to writing the paper and approved the final manuscript.

COMPETING INTERESTS
S.A.A. has received research funding from Incyte. O.B. has received honoraria from Novartis and Sanofi. S.B. is a member of the advisory boards of Qiagen, Novartis, and Cepheid and has received honoraria from Qiagen, Novartis, Bristol–Myers Squibb, Incyte, and Cepheid, as well as research support from Novartis and Cepheid. T.P.H. and S.M. have received honoraria and research funding from Novartis, Pfizer, and Bristol–Myers Squibb (not related to this study).

ADDITIONAL INFORMATION
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41408-022-00667-9.

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