Clonal dynamics using droplet digital polymerase chain reaction in peripheral blood predicts treatment responses in myelodysplastic syndrome

Treatment response evaluation in myelodysplastic syndrome (MDS) and chronic myelomonocytic leukemia (CMML) currently relies on serial assessment of peripheral blood (PB) counts, bone marrow (BM) morphology, and cytogenetics.[1] Acquired genetic alterations are associated with treatment outcomes in MDS[2] and serial assessment of clonal dynamics during therapy could help predict response, refractoriness or progression to acute myeloid leukemia (AML).[3] Most MDS and CMML patients carry somatic driver mutations[2] which can usually be detected in both BM and PB. A good correlation between PB and BM variant allele frequencies (VAF) has been detected in hematological neoplasms.[4,5] Compared to BM sampling, the non-invasiveness of PB as sample source enables more frequent molecular follow-up during treatments, which might be beneficial in several clinical situations, such as in analyzing the role of treatment-induced BM hypoplasia *versus* disease progression as cause of cytopenia during treatment and earlier detection of refractoriness and need for subsequent therapies. Therefore, serial clone size measurement in PB might provide a non-invasive alternative for monitoring treatment responses, including patients whose BM aspirates show hemodilution, dry tap, or those who decline repeat BM sampling.

High sensitivity mutation-specific droplet digital polymerase chain reaction (ddPCR) has been evaluated in AML,[6] but to our knowledge not in MDS patients serially from PB mononuclear cells. As a proof of concept, we evaluated the association between changes in mutational allele burden and treatment outcomes in MDS and CMML using ddPCR of serial PB samples.

Patients who started disease-modifying treatment between January 2016 and October 2018 at Helsinki University Hospital were recruited and those with at least one pathogenic mutation in pretreatment next-generation sequencing (NGS) chosen for follow-up sample collection (17 MDS and 2 CMML patients). Written informed consent was obtained from all patients in accordance with the Declaration of Helsinki. The study was approved by the hospital ethics committee. Serial PB samples were collected before treatment and during routine laboratory visits, and mononuclear cell pellets were frozen for retrospective analysis. Pretreatment BM samples were screened at one time point for myeloid driver mutations as part of the routine clinical workup using an in-house NGS panel of 44 myeloid genes (*Online Supplementary Table S1*). Variants which occurred in the gnomAD database with a frequency of more than 1% were interpreted as normal variation. A variant was interpreted as pathogenic if it was described in the COSMIC database or the variant was a truncating mutation and described in the literature. Targeted mutation-specific wild-type and mutant ddPCR probes were designed and prevalidated by Bio-Rad (www.biorad.com). The QX200 Droplet Generator partitioned the DNA samples (100 ng into 20,000 droplets) for PCR amplification. Following amplification, droplets were divided into negative and positive droplets by setting thresholds on the QX200 Droplet Reader. A mutation was interpreted as positive if at least two positive droplets were detected in each duplicate. Absolute quantification of the target DNA was done by the QuantaSoft Analysis Pro Software (v.1.0, Bio-Rad, Hercules, CA, USA). VAF of 0,1% was used as the lower limit of detection.

One probe each was screened for 11 patients, two probes each for seven patients, and three probes for one patient. The on-treatment PB samples, collected at a median of six time points (range, 4-16) per patient, were tested for altogether 28 patient-specific mutation markers. Statistical analyses were performed using Excel Microsoft 365 and IBM SPSS Statistics 25.

Pretreatment patient characteristics are presented in Table 1 and in the *Online Supplementary Table S2*. The median follow-up between treatment initiation and last ddPCR evaluation was 9.6 months (range, 3.7-39.3 months). Therapy responses were evaluated according to IWG-2006.[1] Eighteen patients were treated with hypomethylating agents (HMA), one with lenalidomide. One patient received venetoclax combined with azacitidine (AZA) in second line. The two CMML patients were treated with hydroxyurea (HU) prior to AZA. Six patients proceeded to allogeneic stem cell transplantation after AZA treatment. The 3-year overall survival (OS) from start of treatment was 42.1% and the median OS was 41 months.

The clonal dynamics in all MDS patients during up to 17 months from start of treatment are shown in Figure 1A, B. The VAF dynamics was associated with the clinical course, both before response and preceding progression. Clinical response (complete remission [CR], marrow CR, or hematologic improvement [HI]) was preceded by

Table 1. Patients' characteristics.

Patient	Age in years/Sex	WHO diagnosis	IPSS-R/ CPSS	Karyotype	Treatment	Best response*	Progression	NGS mutations at diagnosis	Clonal evolution at progression
1	48/M	MDS-EB2	High	t(1;7)	AZA	mCR		*DNMT3A, EZH2*	
2	61/F	MDS-EB2	Intermed	Monosomy 7	AZA	mCR		*DNMT3A, IDH2*	
3	64/M	MDS-EB2	Very high	t(3;3)	AZA	SD	PD	*SF3B1*	NGS and karyotype unchanged
4	71/F	MDS-EB2	High	del(5q)	AZA	HI	AML	*TP53*	NGS unchanged, new chrom 22 loss
5	84/F	MDS-EB2	High	t(1;3)	AZA	CR	Relapse	*SF3B1*	New *CREBBP* Karyotype nd
6	73/F	tMDS-EB2	Very high	del(5q)	AZA	CR	AML	*TP53*	NGS and karyotype unchanged
7	68/M	MDS-RS-MLD	Low	Normal	AZA	SD		*SF3B1*	
8	39/M	MDS-RS-MLD	Intermed	Normal	AZA	SD		*U2AF1,* ***SRSF2***	
9	77/F	tMDS-EB2	High	del(12q)	AZA	mCR	AML	*RUNX1*	New *FLT3* Karyotype nd
10	68/F	MDS-del5q	Very low	del(5q)	LEN	HI		*SF3B1, EZH2*	
11	71/M	CMML-1	CPSS: Intermed	Normal	HU+AZA	HU: SD, AZA: HI	PD	*NRAS, U2AF1*	NGS and karyotype unchanged
12	66/M	CMML-2	CPSS: Intermed	Normal	HU+AZA	mCR		*NRAS, IDH2,* ***SRSF2***	
13	52/M	MDS-EB2	Intermed	Normal	AZA	SD		*TET2 X 2,* ***SRSF2***	
14	77/F	MDS-EB1	Intermed	Normal	AZA	SD		*TET2*	
15	73/M	tMDS-EB2	Very high	del(7q)	AZA	SD	PD	*TET2*	New *PHF6* Karyotype nd
16	67/F	tMDS-EB2	High	del(7q)	AZA+VEN	mCR	AML	*WT1, NRAS, IDH1*	New *NRAS* and *IDH2* Karyotype nd
17	63/F	MDS/ MPN-U	Very high	Complex	DEC	CR	Relapse	*TP53*	New *TP53* Karyotype nd
18	82/M	MDS-EB1	Intermed	Complex	AZA	mCR		*TP53*	
19	69/F	tMDS-EB2	High	Normal	AZA+DEC	mCR		*TP53, DNMT3A*	

*International Working Group-2006 response criteri; bold: no droplet digital polymerase chain reaction (ddPCR) primer. F: female; M: male; WHO: World Health Organization; MDS: myelodysplastic syndrome; MLD: multilineage dysplasia; MPN: myeloproliferative neoplasms; EB: excess blasts; CMML: chronic myelomonocytic leukemia; AML: acute myeloid leukemia; IPSS-R: revised international prognostic scoring system; CPSS: CMML-specific prognostic scoring; mCR: marrow complete remission; NGS: next-generation sequencing; SD: stable disease; intermed: intermediate; del: deletion; CR: complete remission; HI: hematologic improvement; AZA: azacitidine; DEC: decitabine; HU: hydroxyurea; LEN: lenalidomide; PD: progressive disease; VEN: venetoclax; nd: not determined.

decrease in VAF in 91% (10/11) of MDS patients and in 14 of their 15 mutations (median relative decrease in VAF, 76%; range, 35-98%). In patients 1, 2 and 19 the non-*DNMT3A* mutation was chosen for this evaluation.[7] The decrease was observed 33 days (median; range, 0-480 days) prior to the detection of the IWG-2006 response. In particular, we saw a rapid decline in VAF of *TP53* mutations upon AZA treatment initiation in four of five *TP53*-mutated patients (Figure 1A), in accordance with published data.[8]

In 71% (5/7) patients with stable disease (SD), the VAF either decreased slightly or were stable (Figure 1B). Two patients (29 %) had a modest increase in VAF at SD response. These two were the only MDS patients with progression after SD response.

At disease progression after first line treatment (n=9), the VAF of the assumed driver mutations increased in all patients (Figure 1C) although also new clonal findings, in either NGS or karyotype, were detected in six patients at progression (Table 1). The median relative increase in VAF for the initially responded patients (CR, marrow CR or HI) was 293% (range, 42-934%) from maintained response to progression. The VAF increased median 45 days (range, 16-327 days) prior to the clinically verified progression.

Evaluation of subclonal hierarchies using ddPCR was possible in patients with multiple driver mutations. All MDS patients with more than one mutation available for ddPCR monitoring are individually shown in Figure 2. Despite marrow CR response in all three patients with *DNMT3A* mutations, only one patient (patient 1; Figure 2A) had a rapid decrease in *DNMT3A* VAF, whereas the other two patients had only modest decreases in VAF (Figure 2B, C). Serial assessment of the other mutations (*EZH2, IDH2, TP53*, respectively) more closely followed treatment responses in these patients (Figure 2A-C); these results are in line with prior studies showing limited utility of *DNMT3A/TET2/ASXL1* mutations in predicting clonal evolution in AML.[9]

In CMML the response to HMA has been found to be associated with changes in DNA methylation and gene expression, without any decrease in the mutation allele burden, arguing for a predominantly epigenetic effect rather than cytotoxic effect of HMA.[10] In one CMML patient, the VAF followed this pattern (patient 12; *Online*

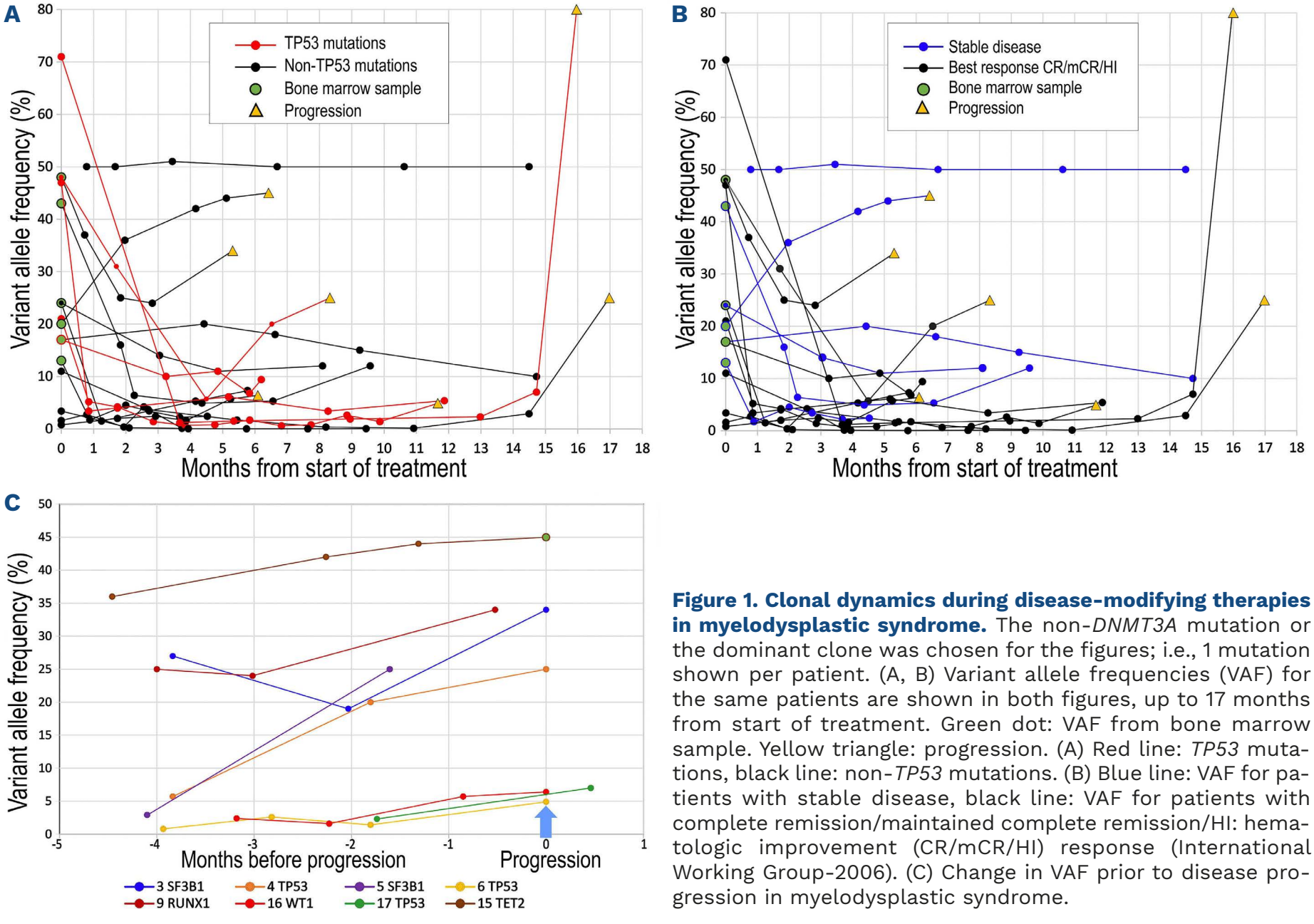

Figure 1. Clonal dynamics during disease-modifying therapies in myelodysplastic syndrome. The non-*DNMT3A* mutation or the dominant clone was chosen for the figures; i.e., 1 mutation shown per patient. (A, B) Variant allele frequencies (VAF) for the same patients are shown in both figures, up to 17 months from start of treatment. Green dot: VAF from bone marrow sample. Yellow triangle: progression. (A) Red line: *TP53* mutations, black line: non-*TP53* mutations. (B) Blue line: VAF for patients with stable disease, black line: VAF for patients with complete remission/maintained complete remission/HI: hematologic improvement (CR/mCR/HI) response (International Working Group-2006). (C) Change in VAF prior to disease progression in myelodysplastic syndrome.

Supplementary Table S3). On the other hand, in our second CMML patient, we saw a dramatic decrease in VAF of both *NRAS* and *U2AF1* mutations during AZA (patient 11; *Online Supplementary Table S3*).

In summary, we found that the clonal dynamics predicted the clinical course at favorable treatment response as well as at progressive disease. The changes in PB mutational burden were seen before the response evaluation with BM samplings, the timings of which were decided by the treating clinicians.

TP53 mutations are associated with poor outcomes in MDS,[11] including post-transplant outcomes.[12] In line with

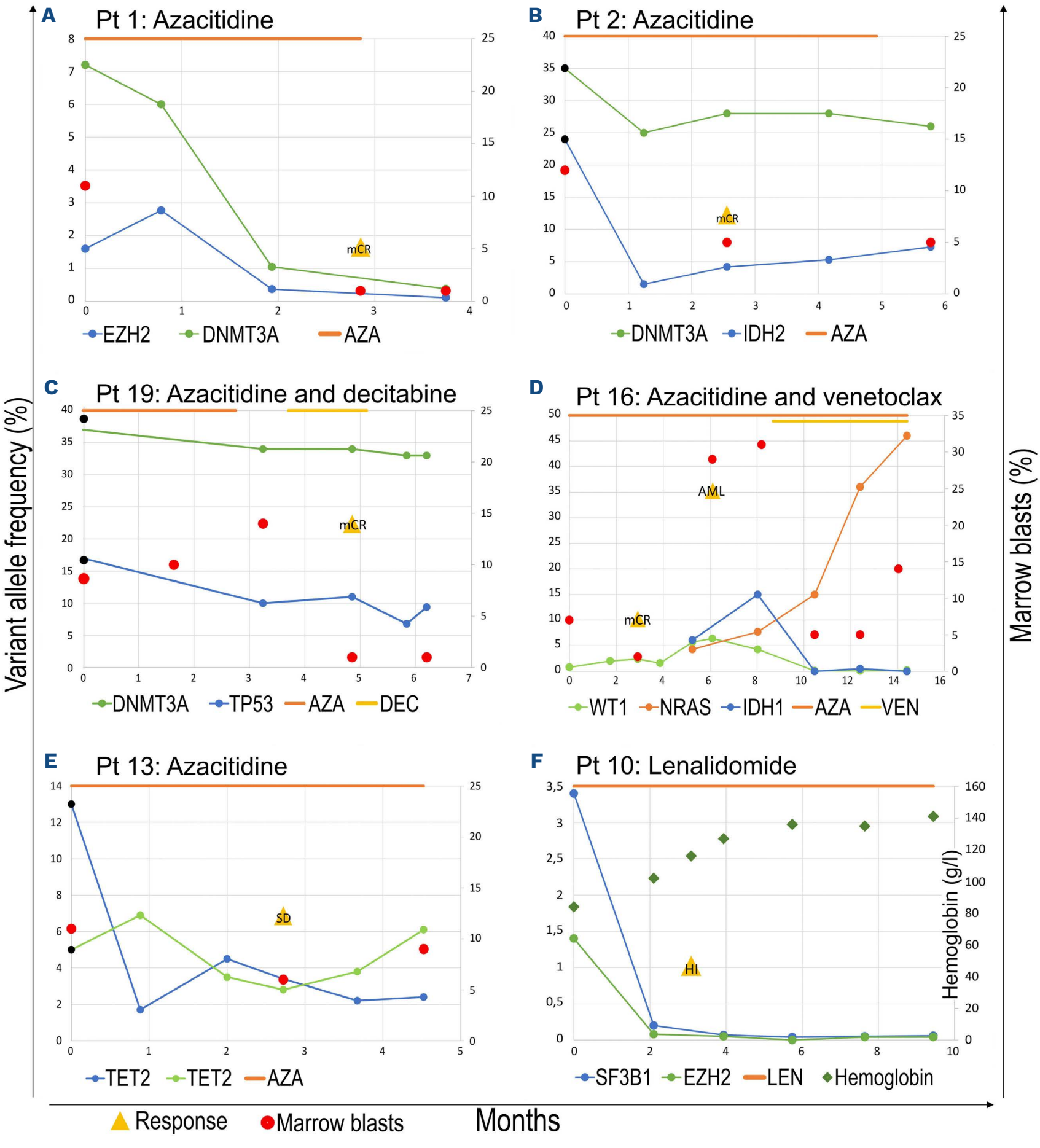

Figure 2. Clonal dynamics and therapeutic responses in all myelodysplastic syndrome patients with more than one pathogenic mutation followed. Peripheral blood variant allele frequencies (black dot: bone marrow) from start of treatment to end of follow-up. Red dots: bone marrow blasts, yellow triangles: therapy response according to International Working Group-2006 criteria. Pt: patient. mCR: marrow complete remission, AML: acute myeloid leukemia, SD: stable disease, HI: hematologic improvement.

a previous study,[8] we observed a rapid decline in clonal burden of *TP53* mutations upon treatment initiation. As reaching minimal residual disease negativity and clearance of *TP53* mutations evaluated by NGS are associated with superior OS in MDS and sAML,[13] serial monitoring of mutational responses in PB may guide clinical decision-making in the future, for example in designing bridging therapies to find the most optimal timing for allogeneic stem cell transplantation.

The DTA mutations (*DNMT3A, TET2* and *ASXL1*) are the most commonly mutated genes in clonal hematopoiesis of indeterminate potential (CHIP) and should usually be excluded as markers for response evaluation, as these mutations may not represent the disease clone.[9] Even some non-DTA mutations in myeloid driver genes may fail to fully capture clonal changes in the BM, and larger studies are needed before implementing single mutation follow-up for assessment of treatment response.[14] Nevertheless the European LeukemiaNet has recommended that if the only detectable mutations are in the DTA genes, these might be used in assessment of residual disease in AML.[15] We saw a robust decrease in VAF of *DNMT3A* in one of the three *DNMT3A* mutated patients. In this study, the three *TET2* mutated patients had SD during AZA treatment, limiting the evaluation regarding the usefulness of these mutations in response evaluation.

Limitations of this study include small cohort size, heterogeneity in patient characteristics and therapies received, as well as the retrospective nature of the analyses. Clonal evolution during MDS progression may occur[3] and PCR-based targeted sequencing is unable to discover new clonal genetic alterations emerging during treatment. Therefore, a new NGS screen should be considered whenever clonal evolution is suspected. Targeted ddPCR is a PCR-based test method and only known sequences can be amplified. The limited number of fluorescence channels and the lack of commercial ddPCR kits limits the evaluation of clonal dynamics to one or, at most, a few mutations per patient. In addition, the short (less than 200 base pairs) probe design is challenging for variants located in homopolymer regions, repeat motifs, as well as for large insertions and deletions.

In conclusion, we found that detection of PB clonal dynamics using patient- and mutation-specific ddPCR may serve as a non-invasive tool for early response evaluation during treatment with HMA in MDS. On the other hand, in CMML, where the treatment response might be more of a stable type, especially during hydroxyurea, this kind of response evaluation might not be feasible.[10] Larger patient series are warranted to evaluate the impact of serial mutational assessment on clinical decision-making and to define the best balance between broad sequencing and more targeted approaches.

Authors

Johanna Illman,[1] Soili Kytölä,[2] Mikko Myllymäki[3,4,5] and Freja Ebeling[3]

[1]Department of Internal Medicine, Porvoo Hospital, Helsinki and Uusimaa Hospital District, Porvoo; [2]Laboratory of Genetics, HUS Diagnostic Center, Helsinki University Hospital and Helsinki University, Helsinki; [3]Helsinki University Hospital, Comprehensive Cancer Center, Division of Hematology, Helsinki; [4]Hematology Research Unit Helsinki, University of Helsinki and Helsinki University Hospital Comprehensive Cancer Center, Helsinki and [5]Translational Immunology Research Program and Department of Clinical Chemistry and Hematology, University of Helsinki, Helsinki, Finland

Correspondence:
J. ILLMAN - johanna.illman@hus.fi

https://doi.org/10.3324/haematol.2022.281595

Received: July 26, 2022.
Accepted: December 15, 2022.
Early view: December 29, 2022.

Disclosures

No conflicts of interest to disclose.

Contributions

JI recruited patients, organized the collection of the study samples, compiled the clinical and molecular data and wrote the manuscript; SK was responsible for implementing the mutational ddPCR analyses in HUSLAB as well as for the NGS and reviewing the molecular data; MM and FE contributed to research discussion and data interpretation; FE was responsible for designing the study. All authors reviewed the manuscript during its preparation and approved the submission.

Funding

This study was supported by grants from the Helsinki University Hospital Comprehensive Cancer Center, the Southern Finland Regional Cancer Center (FICAN South) and from the Finnish Hematology Association. MM was supported by the Sigrid Juselius Foundation, Academy of Finland, and Finnish Medical Foundation.

Data-sharing statement

The authors confirm that the data supporting the findings of this study are available within the article and its *Online Supplementary Appendix*.

References

1. Cheson BD, Greenberg PL, Bennett JM, et al. Clinical application and proposal for modification of the International Working Group (IWG) response criteria in myelodysplasia. Blood. 2006;108(2):419-425.
2. Papaemmanuil E, Gerstung M, Malcovati L, et al. Clinical and biological implications of driver mutations in myelodysplastic syndromes. Blood. 2013;122(22):3616-3627.
3. Makishima H, Yoshizato T, Yoshida K, et al. Dynamics of clonal evolution in myelodysplastic syndromes. Nat Genet. 2017;49(2):204-212.
4. Lucas F, Michaels PD, Wang D, Kim AS. Mutational analysis of hematologic neoplasms in 164 paired peripheral blood and bone marrow samples by next-generation sequencing. Blood Adv. 2020;4(18):4362-4365.
5. Duncavage EJ, Uy GL, Petti AA, et al. Mutational landscape and response are conserved in peripheral blood of AML and MDS patients during decitabine therapy. Blood. 2017;129(10):1397-1401.
6. Parkin B, Londono-Joshi A, Kang Q, Tewari M, Rhim AD, Malek SN. Ultrasensitive mutation detection identifies rare residual cells causing acute myelogenous leukemia relapse. J Clin Invest. 2017;127(9):3484-3495.
7. Gaidzik VI, Weber D, Paschka P, et al. DNMT3A mutant transcript levels persist in remission and do not predict outcome in patients with acute myeloid leukemia. Leukemia. 2018;32(1):30-37.
8. Hunter AM, Komrokji RS, Yun S, et al. Baseline and serial molecular profiling predicts outcomes with hypomethylating agents in myelodysplastic syndromes. Blood Adv. 2021;5(4):1017-1028.
9. 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.
10. Merlevede J, Droin N, Qin T, et al. Mutation allele burden remains unchanged in chronic myelomonocytic leukaemia responding to hypomethylating agents. Nat Commun. 2016;7:10767.
11. Bernard E, Nannya Y, Hasserjian RP, et al. Implications of TP53 allelic state for genome stability, clinical presentation and outcomes in myelodysplastic syndromes. Nat Med. 2020;26(10):1549-1556.
12. Lindsley RC, Saber W, Mar BG, et al. Prognostic mutations in myelodysplastic syndrome after stem-cell transplantation. N Engl J Med. 2017;376(6):536-547.
13. Yun S, Geyer SM, Komrokji RS, et al. Prognostic significance of serial molecular annotation in myelodysplastic syndromes (MDS) and secondary acute myeloid leukemia (sAML). Leukemia. 2021;35(4):1145-1155.
14. Kusne Y, Xie Z, Patnaik MM. Clonal hematopoiesis: molecular and clinical implications. Leuk Res. 2022;113:106787.
15. Heuser M, Freeman SD, Ossenkoppele GJ, et al. 2021 Update on MRD in acute myeloid leukemia: a consensus document from the European LeukemiaNet MRD Working Party. Blood. 2021;138(26):2753-2767.