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Clinical Utility of Next-generation Sequencing in the Management of Myeloproliferative Neoplasms: A Single-Center Experience

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Abstract

Although next-generation sequencing (NGS) has helped characterize the complex genomic landscape of myeloid malignancies, its clinical utility remains undefined. This has resulted in variable funding for NGS testing, limiting its accessibility. At our center, targeted sequencing (TAR-SEQ) using a 54-gene NGS myeloid panel is offered to all new patients referred for myeloid malignancies, as part of a prospective observational study. Here, we evaluated the diagnostic, prognostic, and potential therapeutic utility of clinical grade TAR-SEQ in the routine workflow of 179 patients with myeloproliferative neoplasms (MPN).

Of 13 patients with triple negative (TN) MPN, who lacked driver mutations in *JAK2*, *CALR*, and *MPL*, TAR-SEQ confirmed clonal hematopoiesis in 8 patients. In patients with intermediate-risk myelofibrosis (MF), TAR-SEQ helped optimize clinical decisions in hematopoietic cell transplant (HCT)-eligible patients through identifying a high molecular risk (HMR) mutation profile. The presence of an HMR profile favored HCT in 9 patients with intermediate-1 risk MF. Absence of an HMR profile resulted in a delayed HCT strategy in 10 patients with intermediate-2 risk MF, 7 of which were stable at the last follow-up. Finally, TAR-SEQ identified patients with various targetable mutations in *IDH1/2* (4%), spliceosome genes (28%), and *EZH2* (7%). Some of these patients can be potential candidates for future targeted therapy trials.

In conclusion, we have demonstrated that TAR-SEQ improves the characterization of TN MPN, can be integrated in clinical practice as an additional tool to refine decision making in HCT, and has the potential to identify candidates for future targeted therapy trials.

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Introduction

Over the last decade, the role of characteristic molecular markers in Philadelphia chromosome-negative (Ph^-) myeloproliferative neoplasms (MPN) in both diagnosis and prognosis has been increasingly recognized.[1] Ph^- MPNs are characterized by 1 of 3 classic "driver" mutations identified in the *JAK2*, *CALR*, and *MPL* genes. Testing for these mutations in MPN patients is now considered the diagnostic gold standard. However, a small proportion of patients (~7–10%) with essential thrombocythemia (ET) or primary myelofibrosis (PMF) do not carry the canonical driver mutations.[2] These triple negative (TN) patients may harbor driver mutations that reside in noncanonical sites in *JAK2* and *MPL*,[3,4] or in alternative gene loci. Indeed, in the latest revision of the World Health Organization (WHO) classification of myeloid neoplasms, the authors recommend further genetic testing in this subset of patients to confirm clonality and complement morphologic criteria.[1]

The mutational profile of MPN patients demonstrated prognostic significance in retrospective studies. In PMF, mutations in *ASXL1*, *EZH2*, *IDH1*, *IDH2*, or *SRSF2* predicted shorter survival and/or increased risk of leukemic transformation (LT). The presence of one or more of these mutations defined a higher risk category termed "high molecular risk" (HMR).[5,6] Mutations in *TP53* were also strongly associated with LT despite their low frequency in MPN,[7,8] though *TP53* is not included in the current HMR definition. Our group and others have demonstrated that the presence of an HMR profile, specifically with mutations in *ASXL1* and *EZH2*, is associated with a shorter time-to-treatment failure (TTF) and survival in myelofibrosis (MF) patients treated with JAK1/2 inhibitor (JAKi) therapy.[9–11] These observations may have implications in optimizing the timing of hematopoietic cell transplantation (HCT) in select MF patients. Patients with a higher risk of LT could be considered for early HCT, whereas patients predicted to sustain a durable response with JAKi might benefit from a delayed HCT strategy.

The data are less clear for polycythemia vera (PV) and ET, but emerging evidence suggests that both the number of mutations and the individual mutated genes may be associated with an inferior prognosis.[7,12] The adverse gene mutation profile identified in PV/ET overlaps with, but is not identical to the HMR definition in MF.[12]

Advances in next-generation sequencing (NGS) technology have transformed gene sequencing into a significantly faster and cheaper assay, such that its application in routine clinical practice is now more realizable.[13] Despite the rapid development of NGS technology and the increasing evidence to support the diagnostic and prognostic utility of mutational profiling in MPN, its role in routine clinical decision making is poorly defined. Lack of data on clinical utility has resulted in variable funding of NGS testing in clinical practice. This is exemplified by variable access to NGS testing across Canada, in spite of a universal healthcare system (personal communication, Canadian MPN Group).

At the Princess Margaret Cancer Centre, all new patients referred for myeloid malignancies are offered targeted sequencing (TAR-SEQ) of a panel of 54 genes implicated in myeloid malignancies, as part of a prospective, observational study. Patients can discuss the potential implications of the results on clinical management with the treating physician. In this report, we evaluate the diagnostic, prognostic, and therapeutic utilization of TAR-SEQ in a routine clinical setting for all patients referred with a suspected or confirmed diagnosis of MPN.

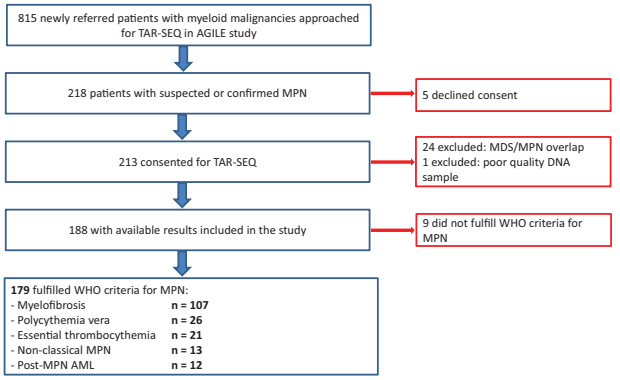

Figure 1. Study patient selection. Flow diagram showing the selection of the study cohort. AGILE = Advanced Genomics in Leukemia, MPN = myeloproliferative neoplasm, MPN/MDS = myeloproliferative neoplasm/myelodysplastic overlap syndrome, TAR-SEQ = targeted sequencing, WHO = World Health Organization.

Patients and methods

Patient cohort

All new patients referred to the MPN/leukemia program at the Princess Margaret Cancer Center were approached for TAR-SEQ testing as part of the Advanced Genomics in Leukemia (AGILE) prospective study. The study was approved by the University Health Network Research Ethics Board. Written informed consent was obtained from all patients, in accordance with the declaration of Helsinki. Patients have the opportunity to discuss the results and any potential impact on clinical management with their treating physician. A total of 815 patients with myeloid malignancies were approached for AGILE consent between February 1, 2015 and November 16, 2016. Two hundred eighteen of these patients were referred specifically for a suspected or proven diagnosis of MPN. One hundred seventy-nine of these fulfilled the 2008 WHO diagnostic criteria for MPN.[14] The selection process of the study cohort is outlined in Fig. 1.

Targeted DNA sequencing and variant classification

DNA samples extracted from peripheral blood (n = 159, 85%) or bone marrow (n = 29, 15%) were used for NGS testing. TAR-SEQ was performed using the TruSight Myeloid Sequencing Panel (Illumina) and run on the MiSeq Illumina platform previously validated by the University Health Network Advanced Molecular Diagnostics Laboratory.[15] Fifty-four genes implicated in myeloid malignancies were profiled (exonic hotspot regions in 39 genes and complete exonic regions in 15 genes, Supplemental Table 1, Supplemental Digital Content, http://links.lww.com/HS/A5) using amplicon-based library preparation as previously described.[9] Data analysis and quality assessment for calling of single-nucleotide variants and short insertions and deletions was performed using NextGene v.2.3.1 (SoftGenetics, State College, PA). Variants detected at coverage >100×, with allele frequency >5% were included for subsequent investigation. Known hotspot or clinically actionable variants detected below these thresholds were verified using orthogonal methods such as Sanger sequencing or digital droplet polymerase chain reaction (ddPCR). Detected variants were then annotated using established criteria[16] as previously reported.[9] Variants of unknown significance were excluded from the analysis of clinical utility. Resulting mutations were classified based on the involved gene as "drivers"

(canonical *JAK2*, *CALR*, and *MPL*), HMR (*ASXL1*, *EZH2*, *IDH1*, *IDH2*, and *SRSF2*), and "oncogenic" (for mutations in other tested genes implicated in myeloid malignancies). Details of all annotated variants are provided in Supplemental Table 2 (Supplemental Digital Content, http://links.lww.com/HS/A5).

Assessment of clinical utility

Patients' TAR-SEQ results were reviewed alongside their clinical information by at least 2 physicians with expertise in MPN. Any comments made by the treating physician in the electronic medical record regarding whether TAR-SEQ influenced the clinical management were taken into account. The impact of TAR-SEQ results on the clinical management was assessed for each patient using a systematic approach. For diagnosis, it was assessed whether TAR-SEQ added evidence to support clonal hematopoiesis in the absence of other molecular or cytogenetic evidence of clonality. For HCT decision making in MF, mutation profiles were deemed high risk if they contained at least one of the previously defined HMR mutations.[5] Cases that had no clear consensus on clinical utility were reviewed in multidisciplinary MPN meetings using the same systematic approach and discrepancies were resolved by consensus.

Results

Patient characteristics and mutational landscape of MPN

The clinical characteristics of the study cohort of 179 patients with Ph$^-$ MPN are outlined in Table 1, including 107 patients

Table 1

Clinical and Laboratory Characteristics of MPN Patient Cohort

	Total	MF (PMF, PPV-MF, PET-MF)	PV	ET	Nonclassical MPN	Post-MPN AML
Number	179	107 (64, 15, 28)	26	21	13	12
Age[a], years, median (range)	66 (19–86)	66 (29–86)	59 (19–75)	55 (26–73)	74 (24–86)	75 (55–84)
Males, n (%)	109 (61)	68 (64)	14 (54)	10 (48)	7 (54)	10 (83)
Hemoglobin, median (range)	113 (65–175)	105 (66–158)	131 (114–175)	138 (115–157)	115 (79–134)	85 (65–129)
Transfusion-requiring, n (%)	39 (22)	31 (29)	0 (0)	0 (0)	2 (15)	6 (50)
Leukocytes, median (range)	9.6 (1.0–151.8)	9.9 (2.1–151.8)	9.8 (4.0–30.0)	6.0 (3.4–13.5)	22.3 (2.8–77.2)	10.3 (1.0–62.9)
Peripheral blasts ≥1%, n (%)	62 (35)	49 (46)	0 (0)	0 (0)	4 (31)	9 (75)
Platelets, median (range)	266 (17–2278)	202 (17–2278)	428 (137–1038)	587 (281–1218)	141 (34–1144)	92 (19–1096)
Cytogenetics						
Evaluable, n (%)	78 (44)	51 (48)	10 (38)	2 (10)	7 (54)	8 (67)
Not done/unsuccessful, n (%)	101 (66)	56 (52)	16 (62)	19 (90)	6 (46)	4 (33)
Karyotype, n (% of evaluable)						
Normal	48 (62)	32 (63)	6 (60)	1 (50)	6 (86)	3 (38)
Abnormal	30 (38)	19 (37)	4 (40)	1 (50)	1 (14)	5 (62)
High risk[b]	8 (10)	4 (8)	0 (0)	NA	NA	4 (50)
Palpable spleen, n (%)	103 (57)	80 (75)	9 (35)	1 (5)	7 (54)	6 (50)
Median palpable, cm, median (largest)	9 (29)	16 (29)	7 (14)	NA	4 (12)	11.5 (18)
Constitutional symptoms, n (%)	41 (23)	32 (30)	1 (4)	0 (0)	5 (38)	4 (33)
History of thrombosis, n (%)	23 (12)	10 (9)	4 (15)	6 (29)	1 (8)	2 (17)
ECOG performance status, n (%)						
0	128 (72)	74 (69)	24 (92)	19 (90)	6 (46)	5 (42)
1	39 (22)	25 (23)	2 (8)	2 (6)	5 (38)	5 (42)
≥2	12 (7)	8 (7)	0 (0)	0 (0)	2 (15)	2 (17)
Transplant-eligible, n (%)	NA	63 (59)	NA	NA	5 (38)	2 (17)
DIPSS risk category[c], n (%)						
Low	NA	11 (10)	NA	NA	NA	NA
Int-1		37 (35)				
Int-2		47 (44)				
High		12 (11)				
PV/ET risk category[d], n (%)						
Low	NA	NA	13 (50)	9 (38)	NA	NA
High			13 (50)	13 (61)		
Mutations, n (%)						
JAK2V617F	112 (63)	69 (65)	20 (77)	10 (48)	5 (38)	8 (67)
JAK2 exon 12	4 (2)	0 (0)	4 (15)	0 (0)	0 (0)	0 (0)
CALR	32 (18)	24 (22)	0 (0)	7 (33)	0 (0)	1 (8)
MPL	12 (7)	9 (8)	0 (0)	3 (14)	0 (0)	0 (0)
HMR[e]	NA	47 (44)	NA	NA	NA	NA
Triple negative, n (%)	13 (8[f])	6 (6)	2 (8)	1 (0.5)	4 (44[f])	NA

DIPSS = Dynamic International Prognostic Scoring System, ECOG = Eastern Cooperative Oncology Group, ET = essential thrombocythemia, HMR = high molecular risk, Int-1 intermediate-1 risk, Int-2 = intermediate-2 risk, MF = myelofibrosis, MPN = myeloproliferative neoplasm, NA = not applicable, PET-MF = post-ET myelofibrosis, PMF = primary myelofibrosis, post-MPN AML = post-MPN acute myeloid leukemia, PPV-MF = post-PV myelofibrosis, PV = polycythemia vera, TAR-SEQ = targeted sequencing.

[a] Age at which TAR-SEQ testing as performed.

[b] High-risk cytogenetics, complex karyotype, or sole or 2 abnormalities that include +8, −7/7q-, i(17q), inv(3), −5/5q-, 12p- or 11q23 rearrangement.

[c] DIPSS uses 5 independent predictors of inferior survival: age > 65 years, hemoglobin < 100 g/L, leukocytes > 25×10^9/L, peripheral blood blasts ≥ 1%, and constitutional symptoms to define risk categories of low, Int-1, Int-2, and high in myelofibrosis.

[d] High-risk disease in PV and ET is defined by age > 60 years or a history of thrombosis. Risk categories were determined relative to the time-point on which TAR-SEQ testing was performed.

[e] HMR profile is defined in MF by the detection of mutations in *ASXL1*, *EZH2*, *IDH1*, *IDH2*, or *SRSF2*.

[f] % of total patients with classical MPN and MPN-unclassifiable excluding patients with other nonclassical MPN and post-MPN AML, n = 163.

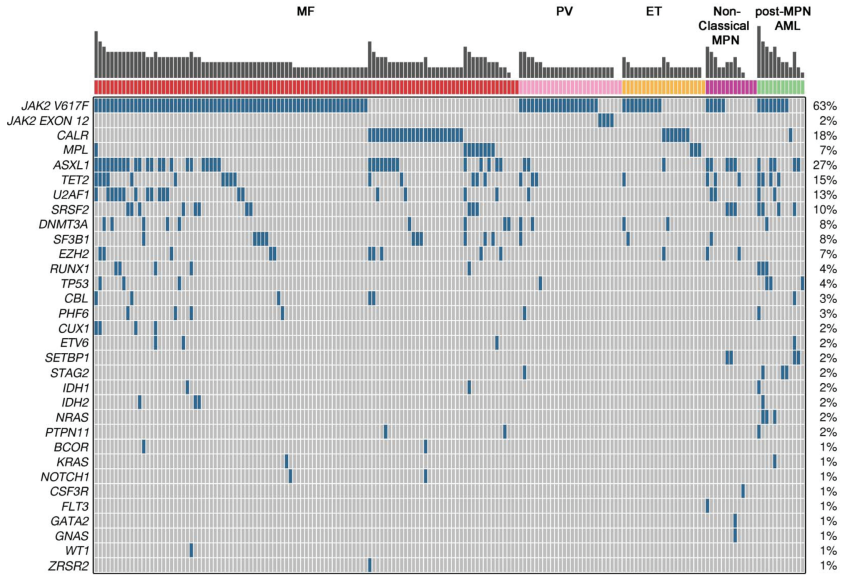

Figure 2. Mutational profile of MPN patients. Landscape diagram demonstrating pathogenic mutations identified in 31/54 sequenced genes (vertical axis). *JAK2* mutations are subdivided into the canonical *JAK2 V617F* mutation (first row) and *JAK2* exon 12 mutations (second row). Each column represents 1 patient sample grouped according to MPN subtype (horizontal axis). Each box shaded in blue represents a pathogenic mutation in the corresponding gene. Upper histogram represents the number of mutations per patient. The column on the right represents the prevalence (%) of each corresponding gene mutation in the patient cohort. ET = essential thrombocythemia, MF = myelofibrosis, MPN = myeloproliferative neoplasm, post-MPN AML = post-MPN acute myeloid leukemia.

with MF, 26 with PV, and 21 with ET. The 107 MF patients comprised 64 patients with PMF, 15 with post-PV MF (PPV-MF), and 28 with post-ET MF (PET-MF). There were 13 patients with nonclassical MPNs including 9 with MPN-unclassifiable (MPN-U), 2 with chronic neutrophilic leukemia, and 2 with hypereosinophilic syndrome. Twelve patients had post-MPN acute myeloid leukemia (post-MPN AML). Pathogenic mutations were detected in 31 out of 54 genes of the myeloid panel (Fig. 2), whereas no mutation was identified in 23 genes.

With respect to driver mutations, *JAK2* mutations were detected in 116 patients (65%). One hundred twelve of these patients carried the canonical *JAK2 V617F* substitution, whereas 4 carried mutations in *JAK2* exon 12. All patients with exon 12 mutations in our cohort had PV. *CALR* mutations were detected in 32 patients (18%) with 18 patients carrying type I/type I-like mutations.[17] *MPL* mutations were detected in 12 patients (7%). The prevalence of *JAK2 V617F*, *CALR*, and *MPL* mutations within each MPN subtype is listed in Table 1. Of 163 patients with classical MPN and MPN-unclassifiable (MPN-U), 13 patients (8%) were identified as TN, with no canonical driver mutations.

Of 107 patients with MF, 47 (44%) had an HMR profile based on harboring at least 1 mutation in *ASXL1* (n = 36, 33%), *EZH2* (n = 10, 9%), *IDH1* (n = 3, 2.8%), *IDH2* (n = 2, 1.8%), or *SRSF2* (n = 11, 10%). Within each DIPSS risk category, HMR profile was detected in 2/11 (22%) patients with low risk, 15/37 (41%) patients intermediate-1 (Int-1) risk, 25/47 (53%) patients with intermediate-2 (Int-2), and 6/12 (50%) patients with high-risk disease.

The median total number of mutations per patient in the study cohort was 2. The median number of mutations was highest in post-MPN AML with 4 mutations followed by MF with 2 mutations, and 1 mutation in both PV and ET. The relative proportion of the number of mutations detected by TAR-SEQ varied across the different MPN subtypes as shown in Fig. 3. The highest proportion of patients with ≥3 mutations were those with post-MPN AML, followed by MF, PV, and ET, respectively ($P < 0.0001$, Fisher exact test). Conversely, ET had the highest proportion of patients with 1 mutation, followed by PV, MF, and post-MPN AML, respectively.

Diagnostic utility of TAR-SEQ: Establishing clonal hematopoiesis in TN MPN

Of the 13 TN patients with chronic phase MPN, TAR-SEQ established evidence of clonal hematopoiesis by identifying mutations in other genes in 8 (62%) patients (Table 2). These included 4 patients with MF (2 with PMF, 2 with PET-MF) and 4

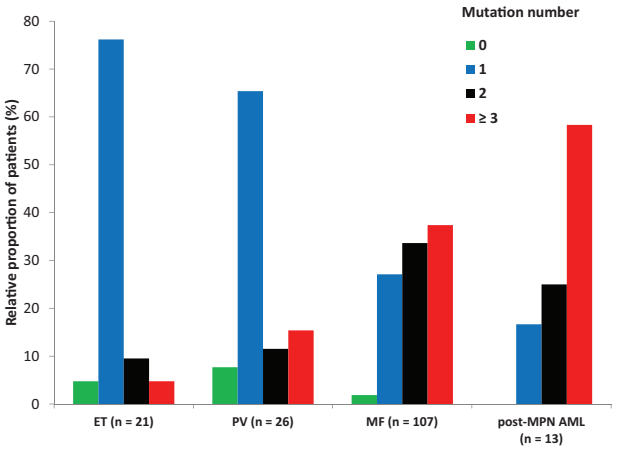

Figure 3. Mutation number in different MPN subtypes. Bar chart representing the number of patients with 0 (green), 1 (blue), 2 (black), or ≥3 (red) mutations detected by TAR-SEQ across the different MPN subtypes calculated as a proportion (%) out of the total number of patients with each MPN subtype listed on the x-axis. ET = essential thrombocythemia, MF = myelofibrosis, MPN = myeloproliferative neoplasm, post-MPN AML = post-MPN acute myeloid leukemia, TAR-SEQ = targeted sequencing.

Table 2

Role of TAR-SEQ in Establishing Evidence of Clonal Hematopoiesis in Chronic Phase Triple-Negative (TN) MPN

Patient ID	MPN	Cytogenetics	Mutations	Evidence of Clonal Hematopoiesis
TN patients in which TAR-SEQ identified mutations in other genes				
18	MPN-U	Normal	*ASXL1 GATA2 GNAS SRSF2*	TAR-SEQ
37	PET-MF	Normal	*NOTCH1 PTPN11*	TAR-SEQ
91	PET-MF	Not done	*DNMT3A*	TAR-SEQ
108	PMF	Normal	*ASXL1 ETV6 U2AF1*	TAR-SEQ
130	PMF	Normal	*ASXL1 EZH2*	TAR-SEQ
159	MPN-U	Not done	*ASXL1 SETBP1 SRSF2*	TAR-SEQ
186	MPN-U	Not done	*ASXL1 NRAS ZRSR2*	TAR-SEQ
188	MPN-U	Abnormal	*EZH2 TET2*	TAR-SEQ abnormal cytogenetics
TN patients in which TAR-SEQ did not identify additional mutations				
19	PMF	Unsuccessful	None	None
57	PET-MF	Unsuccessful	None	None
157	PV	Normal	None	None
175	PV	Abnormal	None	Abnormal cytogenetics
178	ET	Not done	None	None

ET = essential thrombocythemia, MPN-U = myeloproliferative neoplasm unclassifiable, PET-MF = post-ET myelofibrosis, PMF = primary myelofibrosis, PV = polycythemia vera, TAR-SEQ = targeted sequencing, TN = triple negative.

with MPN-U. The 2 patients with PMF had an HMR profile due to the presence of *ASXL1* mutations, whereas the 2 patients with PET-MF had no evidence of HMR. In 7 of the total 8 patients, mutations identified by TAR-SEQ were the only source of evidence of clonality, whereas 1 patient with MPN-U (ID 188) also had abnormal cytogenetics. In the 5 remaining TN patients, TAR-SEQ did not identify any mutations in the tested genes (Table 2).

Optimizing clinical decisions in HCT-eligible patients with intermediate (Int)-risk MF

In our cohort of 107 MF patients, 60 were HCT-eligible (56%) and 47 were HCT-ineligible (44%). The HCT-ineligible patients included 46 patients with age >70 years, prohibitive comorbidities and/or poor performance status, and 1 patient who declined receiving blood products due to religious beliefs. In HCT-eligible patients with DIPSS low- or high-risk disease, TAR-SEQ did not influence clinical decisions. HCT was recommended in all 6 patients with high-risk disease irrespective of their HMR status (present in 4 patients). All 11 patients with low-risk disease were not considered for HCT including 2 patients that had HMR mutations, though these 2 patients were monitored more closely. Of these 2 patients, 1 was lost to follow up and the other remains stable at last follow up.

HMR status influenced clinical decisions in HCT-eligible patients with DIPSS Int risk, specifically in relation to HCT candidacy in Int-1 risk disease and HCT timing in Int-2 risk disease. Of the 27 patients with Int-1 risk disease, 9 (33%) had evidence of HMR mutation status, and were therefore considered for HCT (individual cases summarized in Table 3A). However, HCT was not recommended in 3 of the 9 patients because age >65 years was their only risk factor. These patients were monitored more closely, and HCT was recommended on disease progression as described in patient ID 143 (Table 3A). HCT was recommended in the remaining 6 patients, 5 of whom underwent HCT whereas 1 patient declined. Notably, 4 of the 5 patients who ultimately underwent HCT rapidly acquired additional risk factors, such as a rise in peripheral blood blasts or anemia (Table 3A). In the 18 Int-1 risk patients with no HMR on TAR-SEQ, HCT was not recommended, and these patients were considered for HCT on disease progression.

Of the 19 HCT-eligible patients with Int-2 risk disease, early HCT was recommended in all patients with HMR (n = 9, 47%). By contrast, the absence of HMR in 10 Int-2 risk patients (53%) contributed toward a delayed HCT strategy (individual cases summarized in Table 3B). These patients were also assessed in transplant clinics. In 2 of these patients, HCT was recommended due to the presence of additional risk factors including refractory anemia and thrombocytopenia (ID 68) and 9% peripheral blasts (ID 59). In the remaining 8 patients, HCT was delayed and patients were either observed (n = 4) or treated with non-HCT therapies such as ruxolitinib or hydroxyurea as indicated (n = 4, Table 3B). Seven patients remain stable at last follow up, whereas 1 patient under observation progressed to DIPSS high-risk disease and subsequently underwent HCT (ID 7). The approach to utilizing TAR-SEQ in optimizing clinical decisions in HCT-eligible patients with Int-risk MF is summarized in Fig. 4.

Optimizing risk stratification in PV/ET

In our cohort, 4/26 (15%) patients with PV and 2/21 (10%) patients with ET had 3 or more mutations detected by TAR-SEQ. Furthermore, 1 *JAK2 V617F* positive PV patient (ID 183) was found to be positive for a *TP53* mutation. However, there is no consensus on changing clinical management in PV/ET patients with adverse risk genetic profiles. Therefore, it was decided to monitor these patients more closely.

Potential therapeutic utility of TAR-SEQ

Identifying candidates for potential future clinical trials of novel molecular targeted therapies.

There are a number of novel molecular targeted therapies, which are currently under investigation in hematologic malignancies. These include inhibitors of the spliceosome machinery (eg, H3B-8800), metabolic inhibitors targeting IDH1 and 2, and inhibitors of EZH2, which is implicated in epigenetic modification (Table 4). In our cohort, TAR-SEQ identified multiple MPN patients with corresponding mutations. A total of 50 patients (28%) were found to have spliceosome mutations including *SF3B1* (n = 14, 8%), *SRSF2* (n = 18, 10%), *U2AF1* (n = 23, 13%), and *ZRSR2* (n = 1, 0.6%). Of these patients, 45 were in chronic phase MPN and 5 with post-MPN AML. Seven patients (4%) were identified

Table 3

Utility of TAR-SEQ in Optimizing Clinical Decisions in HCT-Eligible MF Patients With Int-1/2 Risk Disease

Patient ID	Age,[a] years	MF Diagnosis	DIPSS Risk Factors[a,b]	Mutations Detected by TAR-SEQ	Clinical Decision at TAR-SEQ	Comments
(A) Impact of the *presence* of HMR[c] mutation status on clinical decisions in patients with Int-1 risk MF						
10	66	PPV-MF	Age >65	*JAK2*, *EZH2*	Closer monitoring	HCT not recommended as age is the only risk factor
21	67	PET-MF	Age >65	*JAK2*, *ASXL1*, *IDH1*	Closer monitoring	HCT not recommended as age is the only risk factor
52	56	PET-MF	Hb <100	*MPL*, *SRSF2*, *TET2*	HCT	Concomitant chronic lymphocytic leukemia
124	66	PPV-MF	Age >65, PB ≥1%	*JAK2*, *EZH2*	HCT	Rapidly developed anemia progressing to Int-2 risk disease
125	59	PPV-MF	PB ≥1%	*JAK2*, *ASXL1*	HCT	Fluctuating PB 5–8%
126	62	PPV-MF	PB ≥1%	*JAK2*, *IDH2*, *SRSF2*	HCT	Rapidly progressed to accelerated phase with 10% PB
143	67	PMF	Age >65	*JAK2*, *ASXL1*, *RUNX1*, *U2AF1*	Closer monitoring	Closely monitored initially. HCT then recommended on progression to Int-2 risk disease (fluctuating PB at 1–7% and leukocytosis) but patient declined, subsequently enrolled in a clinical trial
155	52	PMF	Symptoms	*MPL*, *SRSF2*, *TET2*	HCT	Rapidly developed fluctuating PB at 4–11%
174	57	PMF	PB ≥1%	*CALR*, *ASXL1*	HCT	HCT recommended but patient declined
(B) Impact of the *absence* of HMR mutation status on clinical decisions in patients with Int-2 risk MF						
5	59	PET-MF	Hb <100, PB ≥1%, symptoms	*CALR*	HCT	HCT recommended as PB were at 9%
7	66	PMF	Age >65, Hb <100	*JAK2*, *TET2*	Observation	Delayed HCT strategy, patient progressed to DIPSS high risk in 12 months and underwent HCT
16	68	PMF	Age >65, Hb <100	*JAK2*, *U2AF1*	HCT	HCT recommended due to refractory anemia and severe thrombocytopenia, but patient declined, treated with supportive therapy
55	57	PMF	Symptoms, WBC >25, PB ≥1%	*JAK2*	Non-HCT therapy	Delayed HCT strategy. Started ruxolitinib, currently stable for 23 months
63	62	PMF	Hb <100, PB ≥1%	*JAK2*, *SF3B1*	Observation	Delayed HCT strategy, currently stable for 18 months
165	70	PMF	Age >65, WBC >25, PB ≥1%	*CALR*, *SF3B1*	Observation	Delayed HCT strategy, currently stable for 13 months
168	68	PMF	Age >65, WBC >25, PB ≥1%	*JAK2*, *SF3B1*	Non-HCT therapy	Already on ruxolitinib at TAR-SEQ acquisition. Delayed HCT strategy and maintained on ruxolitinib, currently stable for 50 months
169	59	PMF	Hb <100, PB ≥1%	*CALR*	Observation	Delayed HCT strategy and started ruxolitinib on development of symptoms. Currently stable on ruxolitinib for 4 months
173	58	PPV-MF	Hb <100, WBC >25, PB ≥1%	*JAK2*	Non-HCT therapy	Already on ruxolitinib at TAR-SEQ acquisition. Delayed HCT strategy and maintained on ruxolitinib, currently stable for 19 months
185	55	PET-MF	Hb <100, PB ≥1%	*JAK2*	Non-HCT therapy	Delayed HCT strategy, started on HU for hyperleukocytosis and currently stable for 7 months

DIPSS = Dynamic International Prognostic Scoring System, HCT = hematopoietic cell transplantation, HMR = high molecular risk, HU = hydroxyurea, JAKi = JAK 1/2 inhibitor, MF = myelofibrosis, PB = peripheral blood blasts, PET-MF = post-ET myelofibrosis, PMF = primary myelofibrosis, PPV-MF = post-PV myelofibrosis, TAR-SEQ = targeted sequencing, WBC = white blood cell. Emboldened genes denote HMR.

[a] Age and DIPSS risk category were determined relative to the time-point on which TAR-SEQ testing was performed.

[b] Abbreviated DIPSS risk factors: age >65, age >65 years; symptoms; constitutional symptoms including fever, drenching night sweats or unintentional weight loss >10% of total body weight over 6 months; Hb <100, hemoglobin <100 g/L; WBC >25, leukocyte count $>25 \times 10^9$/L; PB ≥1%, peripheral blood blasts ≥1% of total leukocyte count.

[c] HMR was defined as mutations in *ASXL1*, *EZH2*, *IDH1*, *IDH2*, or *SRSF2*.

with mutations in *IDH1* or *IDH2*, 5 of which were in chronic phase MPN and 2 with post-MPN AML. Finally, 13 patients (7%) with chronic phase MPN were found to have *EZH2* mutations: 10 with MF, 2 with MPN-U, and 1 with ET. Some of these patients may potentially be considered as candidates for future clinical trials of the novel agents currently under clinical investigation in other hematologic malignancies (Table 4).

TAR-SEQ in the management of post-MPN AML.
In the 12 patients with post-MPN AML, 4 (33%) carried mutations in *TP53*. Five patients (42%) could be potential candidates for future clinical trials of novel targeted therapies due to the presence of mutations in *IDH1*/*IDH2* or in the spliceosome machinery (Table 4). Of note, 3 of the 12 patients died within 6 weeks of diagnosis, and others progressed rapidly. Clinical decisions regarding treatment with chemotherapy or hypomethylating agents were therefore already made in these patients before TAR-SEQ results were available.

Discussion

Our study demonstrates the feasibility of integrating clinical grade genomic profiling using TAR-SEQ in the routine clinical workflow of MPN patients. The ability to perform TAR-SEQ upfront on initial patient referral, along with the high consent rate (98%) enabled us to accurately represent the utility of TAR-SEQ in a "real life" clinical setting. TAR-SEQ showed clinical utility in (a) diagnosis, through verifying clonal hematopoiesis in TN patients; (b) refining clinical decisions relating to HCT in Int-risk MF, and (c) identifying potential candidates for future clinical trials of novel targeted therapies.

Establishing evidence of clonal hematopoiesis through molecular assays in TN MPN has been emphasized in the latest WHO classification of myeloid neoplasms because some patients diagnosed with TN MPN may have a nonclonal disorder of hematopoiesis.[2] Furthermore, there are rare conditions that can mimic the peripheral blood abnormalities and bone marrow

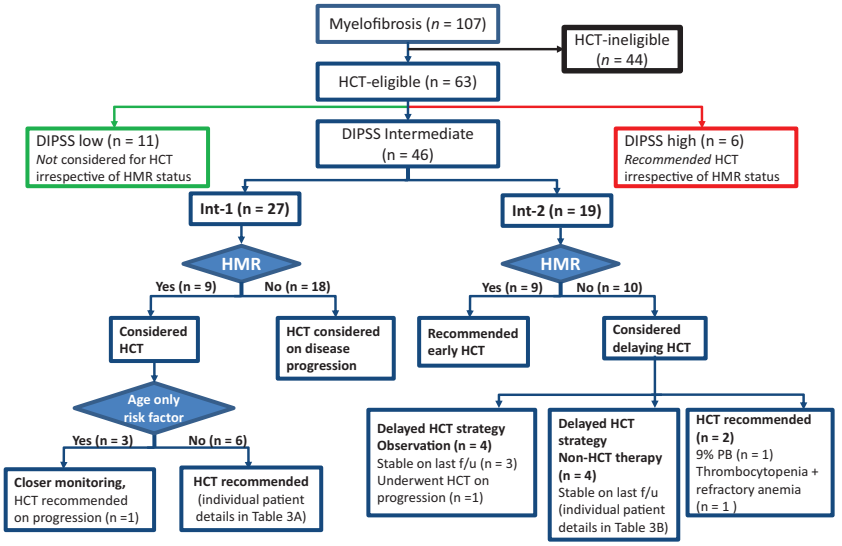

Figure 4. Role of HMR profile as identified by TAR-SEQ in optimizing HCT decisions in MF. Flow diagram showing the utility of identifying an HMR profile in optimizing HCT decisions in HCT-eligible patients with DIPSS intermediate risk myelofibrosis in our patient cohort. DIPSS = Dynamic International Prognostic Scoring System, HCT = hematopoietic cell transplant, HMR = high molecular risk, MF = myelofibrosis, Int-1/2 = intermediate-1/2 risk, TAR-SEQ = targeted sequencing, f/u = follow-up, PB = peripheral blasts.

morphology features associated with MPN, leading to misdiagnosis. We have observed 2 cases of angiosarcoma of the liver with metastases to bone in which bone marrow biopsy was reported as PMF, and subsequent investigations including liver biopsy confirmed the diagnosis. Similar cases have been reported in the literature.[18,19] The presence of a clonal marker would therefore complement the morphological diagnosis of MPN. Conversely, absence of a clonal marker may heighten the suspicion for secondary etiologies or nonclonal disorders, warranting careful evaluation.

There is no consensus on the list of genes that should be screened for mutations in TN MPN. While the WHO recommends screening for the most frequently mutated genes (ie, *ASXL1*, *EZH2*, *TET2*, *IDH1*, *IDH2*, *SRSF2*, and *SF3B1*), given the diversity of mutations occurring in MPN, mutations that establish clonality may occur in other infrequently mutated genes. Of the 8 patients in which TAR-SEQ demonstrated evidence of clonality in our cohort, 2 lacked the most frequent accompanying mutations recommended by the WHO (Table 3). Using an NGS-based gene panel would therefore increase the probability of identifying clonal markers in TN patients. However, despite the broad coverage of commercially available TAR-SEQ panels, there still remain 4 TN patients in our cohort with no clonal markers identified. Diagnostic work up was carefully reviewed in these patients. These patients are being investigated further in a research setting with more extensive sequencing methods such as whole genome sequencing.

Current expert guidelines recommend that clinical decisions in MF should be made based on the DIPSS score, with HCT conventionally offered for patients with Int-2/high-risk disease, and selected patients with Int-1 disease.[20] However, in practice the decision to select HCT versus non-HCT-based therapy is complex and involves careful review of various patient, disease, and HCT-related factors.[21] The wider availability of JAKi, which can help in improving the symptom burden of the disease, has further complicated this decision, particularly the timing of HCT. Furthermore, patients with Int-1/2 risk disease are a heterogeneous population with respect to disease severity and risk of LT, warranting further risk stratification to optimize clinical decisions. NGS is an additional tool that can be integrated in

Table 4

Potential Candidates for Future Clinical Trials of Molecular Targeted Therapies in MPN as Identified by TAR-SEQ

			Available Registered Clinical Trials*		
				Other Hematologic Malignancies	
Mutations	Patients	Molecular Targeted Therapies	MPN	Trial Identifier	Disease Group(s)
Spliceosome machinery (*SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*)	50	H3B-8800 (spliceosome inhibitor)	None	NCT02841540	MDS, AML
Cancer metabolism (*IDH 1/2*)	7	AG-120	None	NCT02074839	AML
		FT-2102		NCT02719574	MDS, AML
		AG-221/Enasidenib		NCT02577406	AML
		AG-881		NCT02492737	AML, advanced MDS
Epigenetic modification (*EZH2*)	13	Tazemetostat	None	NCT01897571	B-cell lymphoma
		CPI-1205		NCT02395601	B-cell lymphoma
		MAK683		NCT02900651	B-cell lymphoma (DLBCL)

AML = acute myeloid leukemia, DLBCL = diffuse large B-cell lymphoma, MDS = myelodysplastic syndrome, MPN = myeloproliferative neoplasm, TAR-SEQ = targeted sequencing.
* Trials obtained from the US National Library of Medicine clinical trial registry www.clinicaltrials.gov.

clinical practice to predict disease-related risk as HMR positive Int-1 risk patients have a shorter survival and/or higher risk of LT making them potential candidates for early HCT (Table 3A). On the contrary, patients lacking HMR may have durable responses to JAKi therapy and can therefore be considered for delayed HCT. Our study showed that several patients with Int-2 risk disease, who lacked an HMR profile on TAR-SEQ had durable responses with JAKi therapy (Table 3B).

In contrast to the heterogeneous patient population with Int-risk disease, TAR-SEQ did not impact HCT decision making in patients with DIPSS high- or low-risk disease. DIPSS high-risk patients are known to have poor prognosis with a median survival of 1.5 years even in the absence of an HMR profile,[5] and a shorter TTF with JAKi therapy (hazard ratio 2.79).[9] Therefore, HCT was recommended for all eligible patients in this risk category irrespective of HMR status. Conversely, the immediate morbidity and mortality associated with HCT likely outweighs the additional risk conferred by the HMR profile in DIPSS low-risk patients, who are typically asymptomatic with a good quality of life.[21] Thus, these patients were not considered for HCT, but patients with an HMR profile were monitored more frequently such that HCT can be considered on disease progression.

Although this study provides a framework for how NGS could be used in practice to inform clinical decisions, the impact of integrating NGS testing on improving the overall outcomes in MF remains unknown, and should be interrogated in well-designed prospective studies. We have developed a framework for integrating NGS in the management of MF for further evaluation in a multicenter study through the Canadian MPN Group (Supplementary Fig. 1, Supplemental Digital Content, http://links.lww.com/HS/A5). Recently, 2 prognostic scores that incorporate mutations in risk stratification for MF are described.[22,23] Clinical integration of mutational profiles in routine clinical work flow will further facilitate the use of these refined scores.

Another advantage of using NGS-based gene panels for risk stratification of MPN over individual gene testing is that the definition of HMR is a "moving target" that will likely further change with time as more data from mutation carriers are collected and validated in different patient cohorts. In addition to *TP53*, an association between adverse outcomes and mutations in several genes outside the current HMR definition including *CBL*, *CEPBA*, *KIT*, *RUNX1*, *SH2B3*, and *TET2* have been reported in retrospective studies,[7,24] warranting further evaluation.

There is no clear HMR definition in PV/ET at present. A case of particular interest in our cohort was a PV patient who carried a *TP53* mutation. Long-term careful follow-up of such patients will help in establishing their clinical course. Mutational profiles may also have implications in predicting thrombosis risk, which is an essential component in the management of PV/ET. A recent study showed that mutations in *DNMT3A* and *TET2* are associated with accelerated atherosclerosis and coronary artery disease in hematologically normal individuals with clonal hematopoiesis of indeterminate potential.[25] It is tempting to speculate that the risk of thrombotic complications in PV/ET patients carrying these mutations may be further increased. Of our 47 patients with PV/ET, 5 patients had mutations in *DNMT3A* and/or *TET2*. Future studies should examine whether these patients have an increased predisposition to cardiovascular thrombotic complications. If proven, this will have a major impact on screening strategies for cardiovascular disease in MPN patients.

Identifying specific genetic mutations amenable to targeted therapy is the cornerstone of personalized medicine. In a significant proportion of our cohort, TAR-SEQ detected mutations in genes that are targets for various molecular-targeted therapies currently under clinical investigation in hematologic malignancies including inhibitors of the spliceosome machinery, *IDH1*, *IDH2*, and *EZH2*. There are currently no targeted therapy trials in MPN, perhaps due to the rarity of these disorders, and a small pharmaceutical market. It is hoped that these data will bring attention to MPN patients for such clinical trials. Investigating novel therapies either as monotherapy or in combination with JAKi is potentially plausible in MF patients who are unlikely to have a durable response with JAKi, or after failure of JAKi therapy. In addition, treatment outcomes for post-MPN AML patients remain unsatisfactory with no improvement in survival made in the last 20 years[26] further encouraging the investigation of novel agents in this poor prognosis patient population.

Finally, it is important to emphasize practical limitations encountered when using NGS that are hindering wider implementation in routine clinical practice. The current process of variant calling is labor intensive, and there is considerable variability in variant annotation between different laboratories. Thus, there is an urgent need to develop standardized criteria for variant classification.[27] Furthermore, current somatic variant classification guidelines do not address finer nuances such as complex interactions between variants within gene pathways. Optimizing turnaround time for test results is also essential, especially in patients with post-MPN AML, who can progress rapidly before the results can be used to inform clinical decision making. The advent of artificial intelligence-enabled variant assessment technologies amenable to machine learning may potentially overcome some of these problems in the future.[28]

In conclusion, this study provides evidence on potential uses of NGS in the routine clinical setting, and provides a framework for integrating NGS in the workflow of MPN. Continuous enrollment in clinical trials or prospective registries is important for further evaluation of the impact of these technologies on improving outcomes of MPN patients.

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