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Integrating mutation variant allele frequency into clinical practice in myeloid malignancies

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Abstract
Hematologic myeloid neoplasms represent a heterogeneous group of disorders with defined clinical and pathologic characteristics. However, intensive investigation into the genetic abnormalities of these diseases has not only significantly advanced our understanding, but also revolutionized our diagnostic and prognostic capabilities. Moreover, more recent discovery on the impact of clonal burden has highlighted the critical and dynamic role of clonal evolution over time, which is integrally linked to a patient's clinical trajectory. This review will highlight the evidence supporting the incorporation of allelic burden of somatic mutations into clinical practice for the diagnosis and prognosis of myeloid neoplasms.

Introduction

Next-generation sequencing (NGS) has brought forth a paradigm shift in the diagnosis, prognosis, and therapeutic management of patients with myeloid neoplasms [1]. Whole-genome and exome sequencing in myeloid neoplasms have led to the near-complete annotation of recurrent mutations in acute myeloid leukemia (AML), myelodysplastic syndromes (MDSs), and many myeloproliferative neoplasms (MPNs), identifying mutations in the vast majority of cases [2–7]. Although current commercial NGS technologies typically report pathogenic mutations that are present at a mutational burden of at least 5%, sufficient sequencing depth allows for mutation detection at frequencies that approach 1% and novel technologies are in development for the identification of variants with much lower variant allele frequency (VAF) thresholds. These technological advances in NGS have led to the identification of rare variants that may be prognostically important and reveal

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previously unidentified intratumoral genetic complexity. Collectively, this has led to several studies that have leveraged genomic data to classify diagnostically relevant subsets of AML, MDS, and MPNs that, in many cases, are both prognostic and predictive of clinically relevant therapeutics.

NGS can quantitate the proportion of variant reads for a given mutation, also known as the VAF, which represents the percentage of tumor cells that harbor a specific mutation assuming a relatively pure leukemic sample. This allows for the digital quantitation of mutational burden; however, to date, the clinical implementation of mutational analysis has largely been of a binary nature with pathogenic mutations being reported only as either positive or negative. Recent studies on myeloid neoplasms have elucidated a novel clinical role of clonal burden/VAF in all facets of the clinical management of these patients. In this review, we will summarize the data that evaluate the clinical utility and implementation of mutational burden in myeloid malignancies.

Next-generation sequencing: the basics

Unlike conventional Sanger sequencing that resolves the sequence of a small genomic region, NGS is a digital technology that sequences large portions of segmented genomic regions in parallel and has been previously reviewed [1,8]. In NGS, by using chip-based approaches, the individual sequence of each genomic region is resolved (or read) and computationally aligned against a reference genome [8]. Mutations or variants are then defined as differences between the reference genome and the aligned reads. Direct comparisons of these variants between a leukemic sample and a germ-line control, or *in silico* approaches, allow for the identification of true somatic mutations.

For clinical NGS, small regions of the genome are interrogated allowing for hundreds, if not thousands, of reads to be mapped to similar regions that allow for the sequential analysis of the same genomic coordinate. Quantification of these variants detected can be simply calculated by dividing the number of reads with the variant against the total number of reads at the genomic position in question. Care should be taken to recognize areas of the genome that are difficult to align or with somatic insertions and deletions as these may be difficult to quantitate.

Allele burden on phenotype and outcomes in MPN

A point mutation in Exon 14 of the Janus kinase 2 gene (*JAK2* V617F) has been identified in the Philadelphia chromosome-negative MPNs, which include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). The mutation (*JAK2* V617F) occurs in approximately 95% of PV patients and 50—60% of ET/PMF patients, as confirmed in multiple studies [9,10]. Importantly, *JAK2* V617F VAF has been found to be tightly coupled to clinical phenotype in MPNs (Table 1) [11]. Specifically, homozygous mutations of *JAK2* V617F, which have VAF > 50%, are significantly more common in PV than in ET (25—30% vs. 2—4%) and associated with higher white blood cell counts, hematocrit, and splenomegaly [12]. In addition, *JAK2* V617F VAF can help differentiate prefibrotic PMF from ET, as a VAF > 50% strongly favors PMF [13].

VAF has also been associated with clinical course in MPNs. In PV and ET, the *JAK2* V617F allele burden identifies a subgroup of patients with a more aggressive phenotype. Specifically, ET patients who are homozygous for *JAK2* have an increased rate of thrombotic events [12]. In both PV and ET, *JAK2* VAF has been associated with a higher rate of fibrotic transformation [12,14,15]. In contrast to ET, the association of *JAK2* allele burden with thrombosis in PV has been controversial. However, one large prospective study reported that patients with a VAF > 75% had the highest risk of thrombosis (approximately 3.5-fold) and were associated with elevated white blood cell counts [16]. Further, a more recent study showed that over time (>5 years after diagnosis), increasing *JAK2* VAF was shown to be the strongest predictor of recurrent thrombosis irrespective of specific MPN diagnosis [17]. In PMF, patients with a *JAK2* allele burden in the lowest quartile had shorted leukemia-free survival (LFS) and overall survival (OS) [18]. The correlation of a low *JAK2* allele burden with inferior outcomes in PMF has been validated in a follow-up study and is independently prognostic in multivariable analysis [19].

Because both studies clinically identified patients with low *JAK2* VAF in a myelodepletive rather than a myeloproliferative state, the negative prognosis of these patients may be driven by the acquisition of additional driver mutations. In support of this hypothesis, targeted NGS of 104 genes in 197 MPN patients evaluated clonal evolution with respect to clinical outcomes and determined these processes to be integrally related [6]. Acquisition of more than one somatic mutation predicted for the risk of leukemic transformation as well as inferior OS and highlighted the deleterious effect of *TP53* clonal expansion. Serial analysis of *TP53* mutation in these MPN patients identified that dramatic VAF increases occur at the time of transformation, thus suggesting that *TP53* VAF may be associated with leukemic transformation in MPNs. Specifically, four patients had serial samples with an initial VAF < 10% in all cases, which was stable for years [6]. Loss of the wild-type (WT) *TP53* allele occurred in three cases followed by rapid clonal expansion (VAF > 50%). Following this clonal expansion, all patients transformed to AML, except one patient who continued to have subclonal *TP53* mutation and did not transform. Further supporting the role of *TP53* in MPN leukemic transformation, alterations of the p53 pathway (either mutation or amplification of chromosome 1q) occurred in 45% of post-MPN AML cases [20]. Amplification of chromosome 1q can inhibit WT p53 expression through overexpression of *MDM4*, a known potent suppressor of p53. In the two cases where samples were available from the chronic MPN stage, both had evidence of the *TP53* mutation in a subclone. Given that multiple studies have shown that the presence of a *TP53* subclone is a predictor of eventual leukemic transformation, this could have important therapeutic ramifications given the propensity for cytotoxic chemotherapies to lead to *TP53* clonal expansion (Figure 1).

In ET and PMF patients who are negative for *JAK2* V617F, point mutations of the thrombopoietin receptor

Table 1 Impact of *JAK2* V617F and *TP53* VAF on MPN.

Diagnosis	Genetic association
PV versus ET	Homozygous mutation in PV
ET versus prefibrotic PMF	*JAK2* VAF > 50% in PMF
Phenotype	
Leukocytosis	↑ *JAK2* VAF
Higher hematocrit	↑ *JAK2* VAF
Splenomegaly	↑ *JAK2* VAF
Outcomes	
Fibrotic transformation (PV and ET)	↑ *JAK2* VAF/homozygous mutation
Thrombosis (ET ± PV)	↑ *JAK2* VAF (>75% in PV)
Leukemic transformation	↓ *JAK2* VAF in PMF (lowest quartile); ↑ *TP53* VAF
Inferior overall survival	↓ *JAK2* VAF in PMF (lowest quartile); ↑ *TP53* VAF

Note: ET = essential thrombocythemia; MPN = myeloproliferative neoplasm; PMF = primary myelofibrosis; PV = polycythemia vera; VAF = variant allele frequency.

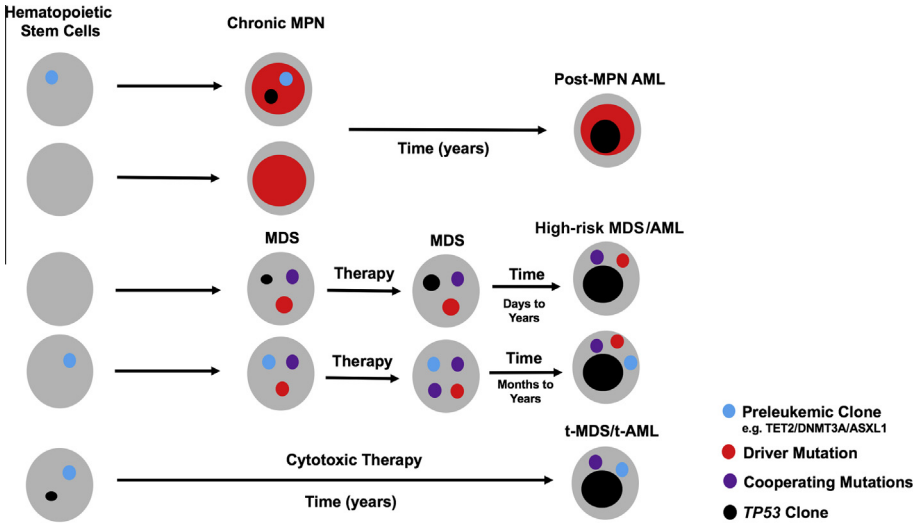

Figure 1 Model for the impact of *TP53* variant allele frequency on disease progression in myeloid neoplasms. Although *TP53* mutation in chronic myeloproliferative neoplasms (MPNs) is a rare event, subclonal mutations can occur. *TP53* clonal expansion strongly correlates with leukemic transformation in MPN. Acquisition of *TP53* mutation is typically a secondary event in myelodysplastic syndromes (MDSs), although it can be present in the preleukemic state, and is tightly coupled with inferior outcomes, which are directly correlated with the clonal burden of *TP53*. Therapy-related MDS and acute myeloid leukemia (AML) likely develops through the potentiation of very rare subclones present prior to treatment with a prolonged latency period. *Note:* t-AML = therapy-related AML; t-MDS = therapy-related MDS.

gene *MPL* (W515L > other variants) occur in 5—10% of cases [21,22]. Although patients with *MPL* W515K mutations had significantly higher VAF than patients with *MPL* W515L, there were no phenotypic differences [22]. Using whole-exome sequencing in *JAK2*/*MPL*-negative ET and PMF patients, recent data have demonstrated that mutations of the calreticulin gene (*CALR*) occur in 67—71% and 56—88% of patients, respectively [23,24]. Mutations of *CALR* are indels and occur nearly exclusively in Exon 9, leading to a mutant protein with a novel C-terminal domain. Approximately 50% of patients have a Type 1 mutation (52 base pair deletion) with 30% of patients having a Type 2 mutation (5 base pair insertion). Importantly, *CALR*-mutant patients were found to have a more indolent clinical course with a lower thrombosis risk and a longer OS in comparison with *JAK2*-mutant patients [23]. Although phenotypic differences among *CALR*-mutant patients have not been described, *CALR* allele burden, as quantified by a fragment analysis assay, could prove to be important for monitoring response in patients treated with interferon alfa [25,26]. Whereas hydroxyurea had no impact on allele burden of *CALR* in ET patients, interferon alfa was able to induce molecular remissions in 42% (13/31) of patients [26]. Of interest, the presence of additional mutations identified by NGS was predictive of clonal remissions, as patients with isolated *CALR* mutations had at least partial molecular remission (VAF reduction > 50%): 13/24 patients with isolated *CALR* mutations versus 0/6 patients with an additional mutation. Whether or not targeting clonal remissions in *CALR*-mutant myeloid neoplasms is clinically important will need to be addressed in prospective clinical trials.

Somatic mutations and risk of acquiring hematologic malignancy

It is now recognized that somatic myeloid mutations are present in individuals without hematologic cytopenias in approximately 5—10% of patients over the age of 65 years and at VAF thresholds that can be identified by commercially available NGS technologies [27—29]. These individuals were characterized by the acquisition of one mutation, on average, which occurred at a relatively low VAF. Although the development of a hematologic malignancy was uncommon (1%/year) in the entire mutant cohort, the risk was 50-fold higher in individuals with a higher clonal burden (VAF > 10%). Overall, these data implicate a direct correlation with clinical trajectories and mutational allelic burden in myeloid neoplasms [28]. Indeed, VAF assessment will likely allow for improved prediction of patients who will ultimately progress to overt hematologic malignancy. To this end, a recent study performed NGS at the time of a non-diagnostic bone marrow biopsy and at definitive progression to MDS and/or AML [30]. The VAF of patients who ultimately developed MDS/AML was significantly increased with a median VAF of 40% versus 9—10% reported in the aforementioned studies with age-related clonal hematopoiesis [27,28]. In addition, patients who developed disease frequently had additional mutations, thus highlighting that the overall mutational burden is a strong predictor for the clinical significance of somatic mutations and may assist in the differentiation of what has recently been termed as clonal hematopoiesis of indeterminate potential from *bona fide* MDS [31]. As proof of principle, a recent report presented a case of a patient who received an allogeneic bone marrow transplantation (BMT) for relapsed lymphoma where both the donor and the recipient developed AML within 4 months [32]. It was subsequently determined that the donor had a preleukemic *DNMT3A* clone with a high allele burden (46%) that ultimately led to the development of two genetically distinct leukemic clones through the acquisition of additional mutations.

Therapy-related MDS (t-MDS) and therapy-related AML (t-AML) have been classically associated with alkylating agents and other cytotoxic therapies and are enriched for aberrations of chromosome 5/7, complex cytogenetics, as well as *TP53* mutations [33—35]. More importantly, we and others have shown that *TP53* mutation is the critical negative prognostic factor as *TP53* mutation patients have inferior outcomes in comparison with even historically poor-risk patients with complex cytogenetics and/or monosomal karyotype [36—38]. The question of the causal relationship between cytotoxic therapy and *TP53* mutation has been advanced. Specifically, Wong and colleagues [39] have recently shown that *TP53* clones (at a very low VAF of 0.003—0.07%) were identified in the peripheral blood or bone marrow in four of seven patients some years prior to the diagnosis of t-AML/t-MDS. Importantly, these rare *TP53* clones were detected in two patients prior to receiving any cytotoxic therapy. These data were further recapitulated in an *in vivo* model, suggesting that patients can be predisposed to therapy-related hematologic neoplasms secondary to hematopoietic stem/progenitor cells harboring age-related *TP53* somatic mutations (Figure 1). Using this novel adaptor sequencing assay with confirmation by droplet digital polymerase chain reaction, the authors were able to show that these very rare variants are found commonly (approximately 50%) in healthy elderly patients. Given the current dismal prognosis of *TP53*-mutant patients, avoiding cytotoxic chemotherapies that could potentiate *TP53* clonal expansion may not only be an important aspect in the treatment of patients with hematologic malignancy, but also in solid malignancy when there are equivalent chemotherapeutic options that do not have this risk. In addition, the presence of rare clones has been found to be prognostically relevant in low-risk MDS patients [40]. Using highly sensitive polymerase chain reaction technology for *NRAS* mutations (variant detection threshold of 0.02—0.1%), the investigators were able to demonstrate in a multivariable analysis that a VAF threshold of 0.5% predicted for inferior outcomes. As technological advances increasingly improve the sensitivity for detecting rare variants, it will be important to redefine their relative impact on the clinical care of patients with myeloid malignancies.

VAF further refines prognostication in AML

Fms-related tyrosine kinase 3-internal tandem duplications (*FLT3*-ITDs) represent the most common mutation in AML (approximately 30%) and are associated with inferior survival in multiple studies [5,41]. The clonal burden of *FLT3*-ITD has been reported to further improve prognostication. Indeed, loss of the WT allele was shown to predict for inferior survival in comparison with *FLT3*-ITD in the heterozygous state [42]. In addition, in the evaluation of prospective clinical trials within the German—Austrian AML Study Group, Schlenk and colleagues [43] identified that a high allelic ratio (AR $\geqslant$ 0.51) of *FLT3*-ITD resulted in inferior complete response rates as well as inferior OS and progression-free survival. More importantly, only the poor-risk high AR subgroup benefited from allogenic BMT, which was confirmed in a multivariable analysis. As the clonal architecture of AML has been increasingly defined, combinations of gene mutations have led to contrasting data on their relative prognostic impact, as has occurred in patients with comutated *FLT3*-ITD and nucleophosmin (*NPM1*) [44]. The mutation load of *FLT3*-ITD is able to elucidate the group of patients who truly have an inferior survival in the context of comutation with *NPM1*. Specifically, only patients with an *FLT3*-ITD of 0.5 or more had inferior event-free survival (EFS) and OS [45]. Furthermore, *FLT3*-ITD as a continuous variable predicted inferior EFS. As an example, patients with an *FLT3*-ITD of 1 had inferior outcomes compared with patients with an *FLT3*-ITD of 0.5—1. In addition, patients with an *FLT3*-ITD < 0.5 did not have inferior outcomes in comparison with *FLT3* WT patients. These data provide evidence that *NPM1*-mutant patients who have *FLT3*-ITD with a ratio < 0.5 also have favorable outcomes and may not benefit from early allogeneic BMT. The aforementioned study confirmed results from the Medical Research Counsel cohort, which also demonstrated that an *FLT3*-ITD threshold of 0.5 was an independent prognostic factor for EFS and OS [46]. However, the exact threshold remains controversial as a previous study identified a threshold of 0.78 to be predictive for LFS and OS [47]. Therefore, future multicenter

Table 2 Impact of clonal burden on phenotype and outcomes in myelodysplastic syndromes.

Phenotype	Genetic association
% of BM ringed sideroblasts	↑ *SF3B1* VAF
Monocytosis	↑ *SRSF2* VAF (*SRSF2*/*TET2* comutant)
Thrombocytopenia	↑ *RUNX1* VAF
Complex cytogenetics	↑ *TP53* VAF
Outcomes	
LFS/OS with HMA	?
LFS/OS with allogeneic BMT	?
Disease progression[a]	↑ *TP53* VAF
OS	↑ *TP53* VAF (>20—40%)

Note: BM = bone marrow; BMT = bone marrow transplantation; HMA = hypomethylating agent therapy; LFS = leukemia-free survival; OS = overall survival; VAF = variant allele frequency.
[a] Progression to higher risk myelodysplastic syndrome or acute myeloid leukemia.

investigation will be required to define the optimal cutoff of *FLT3*-ITD, given the important therapeutic ramifications, particularly its indication for allogeneic BMT in CR1.

The *FLT3*-ITD AR can also be used to predict sensitivity to *FLT3* inhibition, which has been a major focus in recent years to improve the outcomes of this high-risk subgroup. Of interest, primary AML samples with a high AR were significantly more sensitive to *FLT3* inhibition than their low AR counterparts [48]. This has important clinical implications in that *FLT3*-ITD AR is often increased at relapse compared with *FLT3*-ITD AR at diagnosis where *FLT3*-ITD is commonly in a heterozygous/subclonal state [49,50]. Indeed, all relapsed samples or *de novo* samples with high *FLT3*-ITD AR (>50%) were selectively sensitive to *FLT3* inhibitors. These data highlight the concept of *oncogene addiction*, suggesting that clonal burden can be used as a surrogate for the degree of addiction and potentially used for therapeutic personalization. Whether *FLT3*-ITD AR will be an important biomarker to stratify the beneficial effect of *FLT3* inhibition needs to be investigated in future prospective clinical studies.

VAF predicts phenotype in MDS

Recently, highly specific genotype—phenotype associations have been elucidated including *SF3B1* mutation with refractory anemia with ringed sideroblasts and comutations of *SRSF2*/*TET2* with chronic myelomonocytic leukemia (CMML) [2,51—53]. In addition, *SF3B1* VAF strongly predicted for the percentage of ringed sideroblasts, which has been recently confirmed [38,54]. Given the comutation of *SRSF2* or *ZRSR2* with *TET2*, which has a specificity as high as 98.4% for CMML [53], a previous study investigated whether VAF correlated with the degree of monocytosis. Indeed, *SRSF2* VAF directly correlated with both the presence and degree of monocytosis. Although the authors did not find an association with *ZRSR2* VAF, future study is needed as this mutation was only present in a small subset of patients [38].

As multiple studies have associated *TP53* mutation with complex cytogenetics, the possible association between *TP53* VAF and the presence of complex cytogenetics was investigated [38]. Not only did the vast majority of patients with a dominant *TP53* clone have complex cytogenetics (80—100% vs. 40—50% in subclonal *TP53* mutation), *TP53* VAF directly correlated with the number of cytogenetic abnormalities, suggesting that *TP53* clonal expansion is integrally related to genome instability. Finally, we also found an association with the presence and severity of thrombocytopenia with *RUNX1* VAF. As recent studies have identified molecular predictors of response to hypomethylating agent therapy (i.e., *ASXL1* WT/*TET2* mutant) as well as inferior survival with allogeneic BMT [37,55], future investigation is required to determine the impact of VAF on response and outcomes to treatment. Ideally, enhanced understanding of the driver mutations in MDS will ultimately lead to novel targeted therapies. Table 2 presents the molecular abnormalities in MDS that define clinical phenotype and outcomes.

TP53 VAF improves prognostication over binary mutation analysis alone in MDS

In a landmark paper, Bejar and colleagues [7] investigated the prognostic significance of somatic mutations in MDS and discovered that mutations in one of the five genes (*ASXL1*, *EZH2*, *ETV6*, *RUNX1*, and *TP53*) were independently predictive of inferior OS in both univariable and multivariable analyses [7]. More importantly, the presence of any one of these mutations translated into a prognosis similar to the next highest risk International Prognostic Scoring System (IPSS) category, effectively upstaging the prognostic category. In addition, *TP53*-mutant patients had inferior OS to hypomethylating agent therapy and allogeneic BMT, which are the only disease-modifying therapies in MDS [37,55,56]. We have recently investigated the impact of *TP53* VAF on outcomes in myeloid neoplasms [38]. In our training set, *TP53*-mutant patients with a VAF > 40% had a survival of 124 days versus an OS that was not reached in patients with a VAF < 20% (hazard ratio [HR] = 3.52; $p = .01$) and these findings were validated in a clinically independent cohort (HR = 4.94; $p = .01$). Furthermore, *TP53* was predictive of survival as a continuous variable and could further stratify patients independent of their IPSS risk category. In a multivariable model that incorporated age, sex, IPSS category, and allogeneic BMT status, a *TP53* VAF > 40% was the strongest factor predicting inferior outcomes (HR = 1.61; $p < .0001$). Of interest, *TP53*-mutant patients with a VAF < 20% did not have an inferior survival, even in univariable analysis, suggesting that VAF may be the critical factor in identifying the truly high-risk patients. In analysis of the total cohort of *TP53*-mutant patients ($n = 77$), we identified an optimal VAF cutoff point of 20% with a median OS with *TP53* VAF < 20% of 685 days versus VAF of ⩾20% of 183 days ($p = .0036$). Importantly, our study highlighted that *TP53* VAF can have significant prognostic impact at the time of mutation sampling independent of treatment and clinical characteristics. Indeed, recent novel investigations identified the VAF of *TP53* to be the most dramatically expanded clone at the time of progression to AML or higher risk MDS (Figure 1) [57]. The majority of these patients had subclonal *TP53* mutations at diagnosis, and

thus could have important therapeutic implications. However, clonal burden is a dynamic process, and thus a key consideration will be to carry out a future prospective clinical study involving serial analysis of *TP53* VAF and other genes and their impact on survival.

Conclusions

Deciphering the molecular architecture of myeloid neoplasms has greatly enhanced our understanding of these diseases and is now increasingly being used in the clinical management of patients. Investigating the impact of VAF for specific mutations has significantly advanced our understanding on how these mutations drive phenotype as well as refine prognostication. International collaborative efforts are underway to more definitively characterize the impact of VAF in myeloid neoplasms with the goal of developing novel therapies for patients.

Conflicts of interest

The authors have no conflicts of interest to declare.

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