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Original Research Article

Targeted NGS analysis of the canonical genes in 274 Indian patients with suspected myeloproliferative neoplasms: An Indian diagnostic laboratory's perspective

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ABSTRACT

Myeloproliferative neoplasms (MPNs) are caused by somatic pathogenic variants that stimulate increased production and clonal expansion of CD34 multipotent hematopoietic stem cells. Recent World Health Organization (WHO) diagnostic criteria for the diagnosis of Philadelphia chromosome (Ph) negative MPNs includes detection of mutations in the Janus Kinase 2 (*JAK2*), myeloproliferative leukemia (*MPL*), and calreticulin (*CALR*) genes. The purpose of this study was to demonstrate the clinical utility of an in-house next-generation sequencing (NGS) assay targeting only these canonical genes for the molecular diagnosis of patients with Ph-negative MPNs.

We tested 274 samples of patients clinically suspected of having Ph-negative MPNs using an in-house developed NGS panel. The assay consists of two parts, a multiplexed PCR and a highly multiplexed NGS workflow capable of handling diverse samples. The assay is capable of simultaneously detecting mutations in *JAK2* exons 12 and 14, *CALR* exon 9, and *MPL* exon 10.

Of the 274 samples tested, 49 samples harbored mutations in the *JAK2* gene (48 for the *JAK2* V617F and 1 for *JAK2* exon 12), 31 harbored mutations in the *CALR* gene, and two harbored mutations in the *MPL* gene. One sample harbored a mutation each in the *MPL* and *CALR* genes. Here, we present the distribution of mutations in an Indian cohort of 274 patients from India with Ph-negative MPNs. Moreover, we have successfully demonstrated the clinical utility of our in-house multiplexed NGS assay for the molecular diagnosis of MPNs with varying mutation depths

1. Introduction

Myeloproliferative neoplasms (MPNs) are caused by an increased production and clonal expansion of a subset of progenitor stem cells, the CD34 multipotent hematopoietic stem cells.[1] These neoplasms arise owing to somatic pathogenic variants (referred to as mutations for convenience henceforth in this text) that result in myeloid proliferation. MPNs are broadly classified into seven different types: chronic myeloid leukemia (CML) i.e., Philadelphia (Ph)-positive MPNs, polycythemia vera (PV), primary myelofibrosis (PMF), essential thrombocythemia (ET), chronic neutrophilic leukemia, chronic eosinophilic leukemia-NOS and MPN-unclassifiable.[1,2] The classical Ph-negative myeloproliferative neoplasms (MPNs), namely PV, PMF, and ET, harbor driver mutations in the Janus Kinase 2 (*JAK2*),[3–5] myeloproliferative leukemia (*MPL*),[6] and calreticulin (*CALR*)[7,8] genes. The detection of mutations in these genes has been incorporated into the recent World Health Organization (WHO) diagnostic

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criteria for MPNs in conjunction with other clinical, hematological, and morphological findings.[9] 80-90% of Ph-negative chronic MPNs harbor mutations in these genes. At times, subclassifying different MPNs into various subtypes is difficult owing to morphological overlap in disease presentation. Moreover, it is challenging to differentiate between clonal and reactive proliferations. Therefore, the detection of these somatic mutations aids the clinician in patient management. *JAK2* mutations are observed in the majority of cases in the Indian subcontinent;[10] mutations in the *MPL* and *CALR* genes account for 30-40% of Ph-negative chronic MPNs.[6] Targeted capillary (or Sanger) sequencing can be used to check these mutations; however, this method is limited by its relatively low sensitivity (20%) for the detection of MPNs as certain low-depth mutations may be missed.[11] Owing to a need for clinicians to analyze all three genes simultaneously, we have developed an in-house next generation sequencing (NGS) panel that can simultaneously detect mutations in the targeted regions of these three genes and also quantify the relative allele frequency (mutation depth). This provides clinicians with the ability to monitor response to drugs or bone marrow transplantation. Here, we describe the diagnostic utility of this assay for the molecular analysis of clinically suspected MPNs.

2. Materials and Methods

2.1. Patients and samples

Clinical diagnosis: Samples of patients clinically suspected of having Ph-negative MPNs were sent for analysis by the referring hematologist. The clinical features, peripheral blood and bone marrow findings were not available to us.

2.2. DNA extraction

Peripheral blood (2-3 ml) was extracted from individuals with suspected Ph-negative MPN and stored in K2-ethylenediaminetetraacetic acid (EDTA) vacutainers (Becton Dickinson, MD, USA) at 4°C until extraction. Genomic DNA was extracted from 150 μl of blood and eluted in 200 μl of elution buffer using the DNeasy Blood and Tissue Kit (Qiagen, Germany) as per the manufacturer's instructions.

2.3. NGS library preparation

NGS library preparation was performed as described in Ramanan et al.[12] Briefly, NGS primers for targeted regions of *JAK2* exons 12 and 14, *CALR* exon 9, and *MPL* exon 10 were designed using Primer Express v2.0 (Applied Biosystems, CA, USA). Custom "high purity salt-free" primers that contained the target sequences along with NGS adapter tails were synthesized from Integrated DNA Technologies (IDT, IA, USA). A multiplex PCR containing four primer pairs (one primer pair for each: *JAK2* exons 12 and 14, *CALR* exon 9, and *MPL* exon 10) was performed followed by purification and an indexing PCR step. The pooled purified library at a final concentration of 15 pM with 5% phiX (Illumina, CA, USA), was loaded onto an Illumina MiSeq v2 cartridge (Illumina, CA, USA) and run on an Illumina MiSeq next generation sequencer (Illumina, CA, USA) in 2*250 mode. The targeted depth for each region was approximately 5000 reads.

2.4. Bioinformatics

NGS data were analysed using an in-house developed bioinformatics pipeline. The sequences were aligned to the reference GRCH37/hg19 genome and the resulting BAM files were analyzed for variants using the GATK Variant Caller. The BAM and VCF files were visualized using GenomeBrowse v2.1.1 (Golden Helix, MT, USA).

3. Results

3.1. Demographics

In this study, we tested the samples of 274 patients with clinical symptoms indicative of MPNs. Of the 274 patients tested, 190 (69.3%) were male and 84 (30.7%) were female. Eight (2.9%) patients were aged 18 years or younger and 266 (97.1%) patients were above 18 years; 129 (47%) of the 274 patients tested were aged above 50 years.

3.2. Prevalence of JAK2, CALR, and MPL mutations

A detailed distribution of the different mutations detected is shown in Figure 1. The average depth for each region was approximately 3000 reads. The *JAK2* V617F mutation was detected in 17.5% (n = 49) of the suspected 274 samples (representative snapshot shown in Figure 2). The rare J*AK2* exon 12 mutation c.164_1616 delins ATT (H5338Q, K539L) was detected in one patient (0.4%) (Figure 3). 49% of the samples harboring mutations in the *JAK2* gene exhibited mutation depths greater than 50% (Table 1). Among the 274 patients, *CALR* mutations were detected in 31 (11.3%) patients. Type 1 *CALR* mutations[7,13] were observed in 17 patients and type 2 *CALR* mutations were observed in 10 patients (representative images in Figures 4 and 5 respectively). In addition, we observed 12-bp, 31-bp, and 46-bp deletions in exon 10 of the *CALR* gene in one sample each (0.4% each). Of note, 58% of the samples harboring mutations in the *CALR* gene exhibited mutation depths below 20% and only one sample exhibited mutation depth above 50%. Two samples exhibited *MPL* exon 10 mutations (W515L and W515R). Moreover, both mutations were observed at mutation depths below 10%. One patient exhibited co-occurrence of mutations in *CALR* exon 9 and *MPL* exon 10.[14] The *CALR* mutation was a type 1 mutation observed at a mutation depth of 9%. More

than 50% of the positive samples belonged to patients 50 years and above (Table 2). Patients aged above 50 years constituted 87.75% and 64.51% of all *JAK2*- and *CALR*-positive samples respectively.

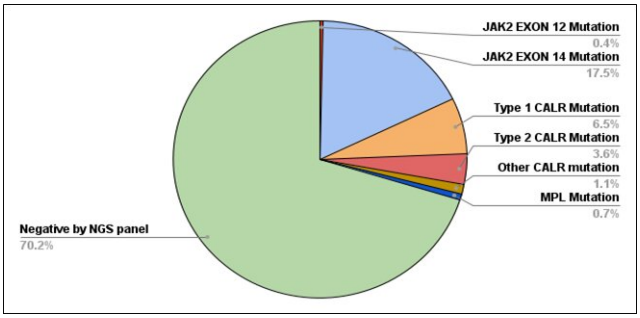

Fig. 1: Distribution of MPN mutations

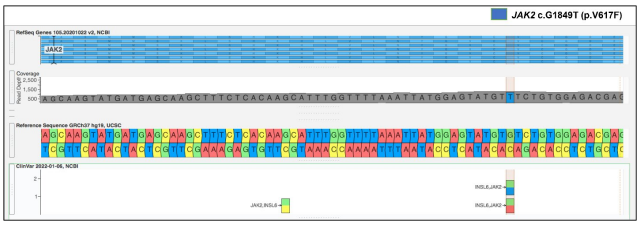

Fig. 2: *JAK2* exon14 snapshot

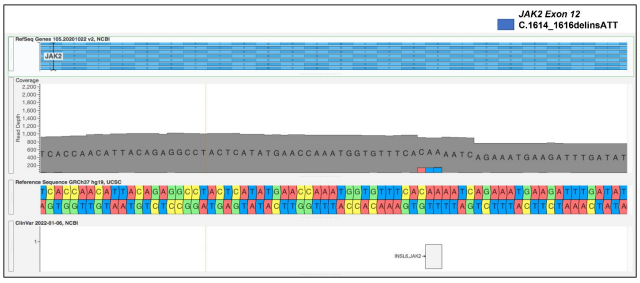

Fig. 3: *JAK2* exon 12 snapshot

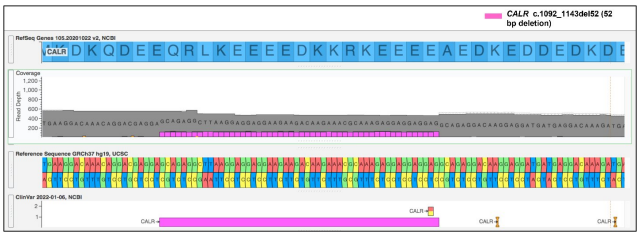

Fig. 4: Type-1 *CALR* mutation (52-bp deletion) snapshot

4. Discussion

BCR-ABL1-negative MPNs are characterized by constitutive activation of the JAK-STAT signaling pathway.

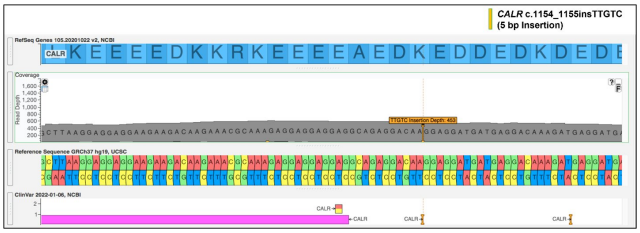

Fig. 5: Type-2 *CALR* mutation (5-bp insertion) snapshot

This is owing to the presence of driver mutations in the *JAK2, CALR*, or *MPL* genes in >90% of these cases.[15] Therefore, mutations in these genes are a major diagnostic criterion for *BCR-ABL1*-negative MPNs in the 2016 WHO classification of myeloid neoplasms.[9] Detecting the presence of these genetic alterations helps in disease diagnosis, which helps prognosis, treatment formulation, and patient management.[16] Various molecular techniques like qPCR, allele-specific PCR, fragment length analysis, and capillary sequencing have been used for the detection of mutations in these genes individually or sequentially in a reflex manner. Use of multiple techniques for the molecular diagnosis of MPNs is not only cumbersome and time-consuming, but also limited by the techniques' inability to detect rare, novel, and low-depth mutations, all of which are generally observed in MPNs. The majority of these assays are targeted to detect canonical mutations only. Moreover, assays like the ARMS PCRs and qPCRs are targeted to detect a specific mutation at a fixed position and may not detect rare or novel mutations within the same region. Therefore, our NGS-based assay was designed to detect both canonical and non-canonical mutations in the reported MPN hotspots— *JAK2* exons 12 and 14, *CALR* exon 9, and *MPL* exon 10.

In a recent study published by Seon Young Kim et al,[17] the authors analyzed the *JAK2, CALR*, and *MPL* genes of 199 patients with MPN. In this study, the authors observed that the *JAK2* V617F mutation was the most frequently encountered mutation (67.3%), followed by mutations in the *CALR* (12.6%), and *MPL* (3.5%) genes. The *JAK2* V617F mutation has been reported as the most frequently encountered mutation in patients with MPN in other studies as well.[18–20] Moreover, a study in an Indian cohort of 50 PMF patients showed that 64% of the patients harbored the *JAK2* V617F mutation, 26% of the patients harbored mutations in the C*ALR* gene, and 2% in the *MPL* gene.[10] Of the 274 samples tested, we observed the *JAK2* V617F exon 14 mutation in only 48 patients (17.5%). One plausible explanation for the low percentage of samples harboring the *JAK2* V617F mutation in our cohort could be that for most of the samples referred to our lab, the hematologists performed the *JAK2* V617F mutation testing elsewhere; only *JAK2* V617F negative samples were referred to our lab to test for other driver mutations by the targeted NGS assay.

Table 1: Mutation depths observed in the *JAK2*, MPL, and *CALR* genes

Mutation Depth	Up to 5%	5 to 20%	20 to 50%	Above 50%
JAK2	4.1% (n = 2)	8.2% (n = 4)	38.8% (n = 19)	49% (n = 24)
CALR	3.2% (n = 1)	54.8% (n = 17)	38.7% (n = 12)	3.2% (n = 1)
MPL	-	100% (n = 2)	-	-

Table 2: MPN positivity by age

Gene	Total number of positives	Percentage of positive samples by age group			
		Up to 30 years	30-50 years	50-70 years	Above 70 years
JAK2	49	4.08% (n = 2)	8.16% (n = 4)	55.10% (n = 27)	32.65% (n = 16)
CALR	31	22.58% (n = 7)	12.90% (n = 4)	51.61% (n = 16)	12.90% (n = 4)
MPL	2	-	50% (n = 1)	50% (n = 1)	-

We detected a rare pathogenic indel, c.1614_1616delinsATT (H538Q, K539L), in *JAK2* exon 12 (Figure 3) in a 28-year-old male patient who presented with portal vein and splenic vein thrombosis with splenomegaly and high Hb levels (17.1 gm/dl). This rare pathogenic indel variant in exon 12 results in an amino acid change from histidine to glutamine at position 538 (H538Q) and from lysine to leucine at position 539 (K539L). It is generally observed in cases of polycythemia vera and primary erythrocytosis with low serum erythropoietin levels. Of the 274 samples tested, 193 patients (70.2%) did not exhibit any driver mutations in the *JAK2, CALR,* and *MPL* genes. The predominance of triple-negative MPNs (over *JAK2* V617F mutation) in our study could be multifold. It is possible that some of the patients clinically suspected of having an MPN did not have a clonal myeloproliferation and the clinical symptoms such as the increase in Hb, WBC, and platelet counts was owing to secondary or reactive conditions. Secondly, the clinical MPN symptoms could be caused by mutations in genes other than the three targeted in this study. Grinfield et al sequenced the exonic regions of 69 genes implicated in myeloid cancer from more than 2000 patients. Notably, their study showed that genes other than *JAK2, MPL*, and *CALR* were implicated in more than 55% of the cases.[21] A plethora of somatic mutations in genes implicated in MPNs and other myeloid malignancies have recently come to the fore as a result of extensive NGS-based studies.[15] Importantly, these mutations have also been reported in cases of clonal hematopoiesis of indeterminate potential (CHIP), which makes these genes ideal candidates for a targeted NGS assay capable of detecting low-depth mutations. These genes have diverse functions such as DNA methylation (IDH1/2;[22]) histone modification (ASXL1;[23]), mRNA splicing (SRSF2;[24]), and cell signaling (NRAS/KRAS;[25]).

An NGS assay helps ascertain the depth of a particular mutation, which helps the clinician understand the disease burden in terms of percentage of cells that are affected by the mutation. Our NGS assay can detect mutations at depths as low as 0.5%[12] whereas capillary sequencing can detect mutations reliably at depths of only 15-20% and above.[11] Higher sequencing depth increases the accuracy of a sequencing assay for detecting low allele burden mutations.[26] Almost half the samples harboring mutations in the *JAK2* gene exhibited mutation depths greater than 50%; however, more than 90% of the samples harboring mutations in the *CALR* gene exhibited mutation depths lower than 20%. Moreover, both the samples harboring mutations in the *MPL* gene exhibited mutation depth lower than 20%. This highlights the diagnostic utility of an NGS-based assay as it can help in the diagnosis of cases where the patients are harboring driver mutations with low mutation depths.

A major limitation of our study is that we do not have the clinical data for all the samples tested by our in-house MPN NGS. Integration of the clinical data with the NGS results would give us a much deeper insight into the prevalence of driver mutations in the *JAK2, CALR*, and *MPL* genes in individuals with different MPN subtypes in the Indian population. However, here, we aimed to demonstrate the utility of our NGS assay for the molecular analysis of the three most-commonly implicated genes in Indian patients with suspected MPN. This panel has great utility in a resource-limited setting with financial constraints where patients cannot afford elaborate panels. Additionally, our results indicate the need for a larger panel that takes into account other genes that have been associated with MPNs.

5. Source of Funding

None.

6. Conflicts of Interest

The authors declare no conflicts of interest.

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