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The clinical utility of a custom-developed targeted next-generation sequencing assay for detection of mutations associated with Philadelphia-negative chronic myeloproliferative neoplasms: Two case examples with *CALR* exon 9 mutations

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ABSTRACT

We have developed a highly targeted custom next generation sequencing-based test targeting *JAK2* exons 12 and 14, *CALR* exon 9, and *MPL* exon 10, which are implicated in Philadelphia-negative chronic myeloproliferative neoplasms. The assay is capable of ultra-high sequencing depth and producing mutational detection sensitivities of 0.5% and below. We have validated the performance of the test through orthogonal testing and demonstrated a high degree of multiplexing with up to 50 samples in a single run. We show the clinical utility of this test through the description of a couple of cases with myeloproliferative disorders with Type-II and Type-II-like mutations in exon 9 of the *CALR* gene.

Key words: NGS, multiplexing, CALR, JAK2, Hemato-oncology, Philadelphia-negative chronic myeloproliferative neoplasms

Introduction

The completion of the first draft of the reference human genome took nearly 13 years (between 1989 and 2002), and involved thousands of scientists across the globe working on thousands of capillary sequencing instruments at an overall cost of more than US$ 3 billion.[1] Today, <15 years after the completion of that landmark draft, it is possible for a single scientist to fully sequence a human genome in a couple of days, at a cost that is rapidly approaching US$ 1000. This remarkable advance, which has significantly outpaced the highly cited Moore's Law in the field of semiconductors, has been made possible through the advent of a series of technologies collectively referred to as next generation sequencing (NGS) or massively parallel sequencing.[2,3]

A detailed technical description of NGS technologies is beyond the scope of this article, and readers are directed to several excellent reviews that have been published in recent years.[2,3] To give context to the discussion ahead, however, we provide a very brief outline of the steps involved in the NGS workflow. The shared basis of the most NGS technologies is the generation of gigabases of sequence data per run through the parallel execution of millions of sequencing reactions of relatively short read length (30–500 bp). The steps in most NGS approaches consist of (i) fragmentation of the DNA to be sequenced, (ii) addition of adaptor molecules to the ends of the fragments for priming of amplification and sequencing reactions and incorporation of multiplexing barcodes, (iii) clonal amplification of the fragments, (iv) real-time sequencing of the fragmented pieces, (v) acquisition of raw data, (vi) conversion/deconvolution of the raw data into nucleotide base calls followed by assembly/alignment and variant calling, and (vii) filtering of variants and generation of clinically relevant report.

NGS has enabled a number of applications that were hitherto impossible or impractical. These include whole genome resequencing, *de novo* genome sequencing and targeted sequencing of whole exomes, partial exomes, large targeted genes, and gene panels. These applications have revolutionized the identification of genomic variants in germline mendelian disorders as well as in somatic cancer tissue and have facilitated unprecedented advances in the analyses of complex phenotypes.[2] The NGS technique of incorporating multiplexing adapter barcodes allows for the simultaneous analysis of multiple samples at a significantly decreased cost. The ultra-high sequencing depth possible through NGS has allowed the detection of variants present at low frequency in mosaic states, low-level somatic mutations, and novel applications such as noninvasive prenatal diagnostics through analysis of circulating fetal DNA and noninvasive tumor diagnostics from cell-free circulating tumor DNA.

Yet, in spite of all this progress, access to NGS in routine clinical practice remains very limited. There are a number of reasons for the lag in adoption. The core and ancillary

instrumentation required for NGS is expensive and its operation requires highly trained interdisciplinary teams. The preanalytical steps of library preparation are extremely complex and, more often than not, require the use of expensive proprietary reagents. Equally challenging are the postanalytical steps of data analysis, data storage, and clinical interpretation of results which require expensive hardware, software, and cross-disciplinary expertise. In response to these challenges, commercial vendors have been forced to develop large "one glove fits all" type of gene panel kits and analytical pipelines that can cater to a wide variety of applications in a standardized format. While this makes NGS more accessible, in spite of a logarithmic reduction in a base cost of DNA sequencing, the cost of a test to the end-user has not decreased. Furthermore, end users are faced with a deluge of data that may not be relevant to the condition, and at worst may confuse the diagnosis. What is needed is the wide availability of highly targeted clinically relevant panels that offer all the advantages of NGS without the limitations highlighted above.

We have developed a highly targeted multiplexed NGS assay for clinical use in the area of hemato-oncology. The fields of hematology and hemato-oncology have historically been early adopters and champions of molecular diagnostic technologies. The identification of the Philadelphia chromosome and the use of tyrosine kinase inhibitors (TKIs) in chronic myeloid leukemia (CML) are a poster story for the use of the molecular technique in diagnosis, monitoring, assessing response to treatment, and use of second-line TKIs in resistant patients.[4] Mutations in the *JAK2*, *MPL,* and *CALR* genes are found in 80–90% of Philadelphia-negative chronic myeloproliferative neoplasms (CMPNs) which include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF).[5] Conventional molecular techniques for comprehensive analysis of these genes include sequential capillary sequencing of these genes, which is time-consuming, labor intensive and has limited throughput. NGS offers several advantages in terms of multiplexing capacity, ultra-high throughput, and ability to provide mutational signatures/backgrounds in single experiments and has the potential to suggest targeted therapies at the time of the diagnosis. Here, we describe the development and utility of a targeted multiplexed custom amplicon based NGS assay for Philadelphia-negative CMPN targeting exons 12 and 14 of *JAK2*, exon 10 of *MPL,* and exon 9 of the *CALR* gene. This assay is highly sensitive, specific and more affordable than existing assays.

Materials and Methods

Samples and DNA extraction

Two to three milliliters of peripheral blood from Philadelphia negative CMPN patients was collected in K_2-ethylenediaminetetraacetic acid (EDTA) Vacutainers (Becton Dickinson, MD, USA). Samples were stored 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 instructions in the kit insert.

Assay design

Primers for amplification of *JAK2* exons 12 and 14 were designed *de novo* using Primer Express v2.0 (Applied Biosystems, CA, USA). The primers for amplification of *CALR* exon 9 and *MPL* exon 10 were designed using the same software based on binding sites described in the literature.[6,7] The primer binding sites were selected to ensure nonoverlap with known mutations in the COSMIC database. The primers were tailed with common forward and reverse in-house 20 bp tags on their 5' ends to facilitate the use of common sequencing primers for capillary sequencing (CE-seq) and adapter ligation for NGS. Primers for real-time SYBR green amplification refractory mutation system (ARMS) polymerase chain reaction (PCR) for the detection of the V617F mutation in exon 14 of *JAK2* were also designed using Primer Express v2.0 with manual modifications to make them stringently allele specific for the mutation. For NGS library preparation, long indexing adapter primers (~87–101 bp) with the following structure from 5' to 3' end were designed: P5/P7 binding sites (for binding to the Illumina flow cell) - i5/i7 index (8 nucleotide barcodes for multiplexing) - P5/P7 sequencing primer binding sites (for binding of the Illumina sequencing primers) - optional diversity spacer (to enhance optical cluster separation) - common in-house forward/reverse tags as described above (to enable amplification and adapter addition to the earlier generated amplicons). Custom primer synthesis was carried out from Integrated DNA Technologies (IDT, IA, USA), Eurofins (Luxembourg) and Sigma (India). All primers were ordered "standard desalted" except for the long NGS primers which were ordered "high purity salt free."

Polymerase chain reaction

PCRs for *JAK2* exons 12 and 14, *CALR* exon 9, and *MPL* exon 10 were carried out as uniplex reactions for assay optimization and capillary sequencing using conventional PCR (Veriti and SimpliAmp Thermo Cylers, Applied Biosystems, CA, USA) as well as real-time SYBR Green PCR with melt curve analysis (Illumina Eco, CA, USA; Qiagen Rotor-Gene Q, Germany; BioRad CFX 96, CA, USA) to establish the sensitivity and specificity of the assays. Uniplex PCRs were carried out at a final volume of 20 µl in a buffer containing 1X 5 Prime HotMaster Taq 10X buffer (5 Prime, CO, USA), 0.2 mM dNTP (Illumina/Epicentre, WI, USA), 0.1 units of Platinum Taq Hot Start Polymerase (Invitrogen, CA, USA), 0.1 units of HotMaster Taq (5 Prime, CO, USA), and 200 nM final primer concentrations for forward and reverse primers each; optionally during optimization 0.35X final concentration SYBR Green (Invitrogen, CA, USA) was used. Cycling conditions were initial denaturation for 5 min at 95°C followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 63°C for 25 s, extension at 72°C for 35 s, and final extension at 72°C for 10 min. For multiplexed reactions of all four

amplicons together (used for NGS library preparation), the same cycling conditions were followed with some variations. The QuantiTect Multiplex PCR Kit (Qiagen, Germany) was used in place of the individual PCR components, and the primer concentrations were reduced to 50 nM each. For real-time SYBR green ARMS PCR, two parallel reactions for the wild type and mutant V617F type reactions and were run with a common reverse primer in both tubes and allele specific forward primers in each tube. Reaction setup was similar to the conditions described for the uniplex reactions above, with the use of 300 nM final concentration of each primer and addition of 0.45X SYBR Green (for real-time monitoring) and 0.2 mM dUTPs (Illumina/Epicentre, WI, USA) to minimize carry-over contamination. PCR conditions comprised initial denaturation at 95°C for 2 min, followed by 38 cycles of 95°C for 15 s for denaturation, 57°C for 20 s for annealing along with fluorescence acquisition on the FAM channel, and extension at 72°C for 15 s. Cycling was followed by melt curve analysis which involves fluorescence acquisition during a temperature ramp from 65°C to 95°C in 0.5°C increments.

Capillary sequencing

Uniplex PCR products were checked by gel electrophoresis (Lonza, Switzerland) and purified using the Purelink PCR Purification Kit (Invitrogen, CA, USA). DNA was quantified using the Qubit system (Life Technologies, CA, USA). The dideoxy sequencing reaction was run using the BigDye Terminator Cycle Sequencing Kit v3.1 (Life Technologies, CA, USA) with standard cycling conditions as described in the product insert on an ABI SimpliAmp Thermo Cycler (Life Technologies, CA, USA). Cycle sequencing products were purified using Ethanol-EDTA-Sodium Acetate Precipitation (Merck, Germany - Sigma, India), denatured with HiDi Formamide (Life Technologies, CA, USA) for 5 min at 95°C, and cooled on ice for 5 min. Capillary electrophoresis of the sequencing products was carried out on an ABI 3500 Genetic Analyzer (Life Technologies, CA, USA) with the following configuration: POP-7 polymer, 50 cm capillary and standard loading conditions as described in the sequencing kit and instrument manual.

Library preparation and next generation sequencing

Multiplexed PCR products of *JAK2* exons 12 and 14, *CALR* exon 9, and *MPL* exon 10 from each individual patient were purified using the Purelink PCR Purification Kit (Invitrogen, CA, USA). DNA was quantified using the Qubit system (Life Technologies, CA, USA). PCR products were diluted 1/10 using 10 mM Tris-HCl pH 8.0, and used as templates for the subsequent indexing and adapter addition PCRs. For indexing PCR, the QuantiTect Multiplex PCR kit was used as above. Indexing primers were used at 200 nM. Cycling conditions comprised initial denaturation at 95°C for 15 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 63°C for 45 s, and extension at 72°C for 90 s. The ramp rate was reduced to 1.5°C/s during this cycling. Final extension was at 72°C for 10 min. Products were quantified using the Qubit system; when multiple samples were used, concentrations were molar normalized before pooling. Pooled samples were re-purified using the Purelink PCR Purification Kit and diluted down to 4 nM final concentration using resuspension buffer (RSB - Illumina, CA, USA). Samples were denatured for 5 min using 0.2 N NaOH and neutralized using HT1 buffer (Illumina, CA, USA). At this point, samples were pooled with other libraries prepared for NGS in a ratio dependent on amplicon size/total panel size, desired sequencing depth, and number of samples pooled in each sub-library. Pooled libraries were further diluted down to a final 15 pM library that included a 5% phiX library spike (Illumina, CA, USA) as a control and diversity enhancer. The samples were loaded onto an Illumina MiSeq v3 cartridge (Illumina, CA, USA) and run in 2*300 mode on an Illumina MiSeq next generation sequencer (Illumina, CA, USA).

Data analysis

For capillary sequencing, the .ab1 files generated on the ABI 3500 Genetic Analyzer were analyzed manually using 4 peaks for Mac OS (Nucleobytes, The Netherlands) and automatically using Mutation Surveyor v5.0 (Softgenetics, PA, USA) and SeqScape v3.0 (Life Technologies, CA, USA) on Windows 7. For NGS, raw .fastq sequence files generated by the Illumina MiSeq instrument were uploaded to Illumina's cloud analysis portal - BaseSpace (Illumina, CA, USA). Alignment to the reference genome (hg19) was carried out using the Burrows Wheeler Aligner (BWA), and the resulting.bam files were analyzed for variants using the GATK Variant Caller (BaseSpace BWA Enrichment Workflow v2.1.1. with BWA 0.7.7-isis-1.0.0 and GATK v1.6-23-gf0210b3). Visualization of .bam and .vcf files was carried out using GenomeBrowse v2.1.1 (Golden Helix, MT, USA).

Results

Assay development

The specificity of the uniplex *JAK2* exon 12 and exon 14, *CALR* exon 9, and *MPL* exon 10 assays was established through melt curve analysis, gel electrophoresis and bidirectional capillary sequencing of amplified product followed by alignment of sequence data to a reference genome. Bidirectional capillary sequencing of healthy samples during the development phase produced sequence data with 100% homology to the target regions on the reference genome assembly. Once the uniplex assays were validated, a multiplex reaction was created and optimized for NGS. During multiplex assay development, we tested 88 CMPN samples including some previously detected *JAK2* V617F positive samples using our custom NGS assay. The results for the NGS assays were validated using real-time ARMS PCRs for previously unconfirmed *JAK2* V617F mutation and using bidirectional capillary sequencing for the other targets [Figure 1] (for an example of orthogonal results for the V617F mutation). No discrepant results were produced during orthogonal testing. In a single run, we have multiplexed up to 50 samples [Figure 2] (for a multiplexed run with

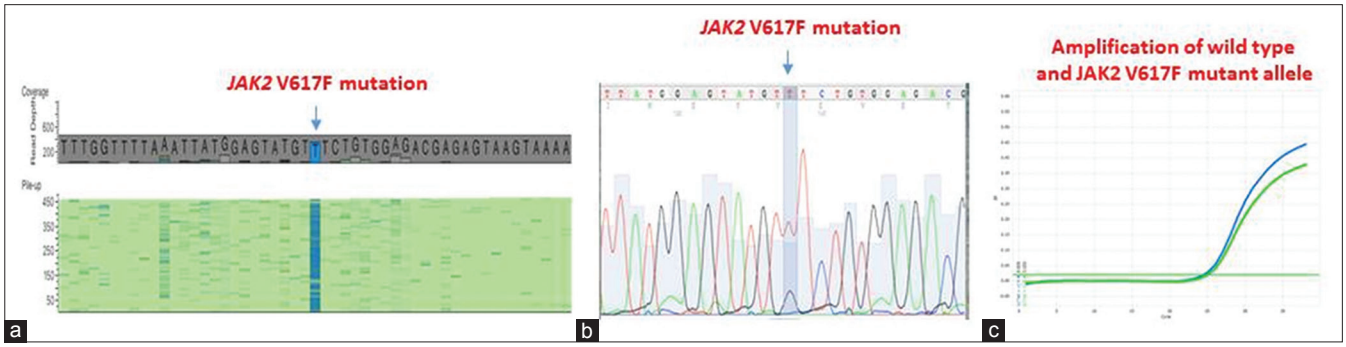

Figure 1: Orthogonal confirmation of the *JAK2* V617F mutation (G>T) by: (a) Next-generation sequencing data visualized in GenomeBrowse, (b) capillary sequencing trace, and (c) real-time amplification refractory mutation system polymerase chain reaction curves. The next-generation sequencing data pane shows, from top to bottom, the coverage, alignment of sequence reads, and reference genome DNA sequence along with the translated protein sequence. The capillary traces show the mixed base G>T change in the forward direction. The real time amplification refractory mutation system polymerase chain reaction curves show amplification for both the wild type allele as well as the mutant allele

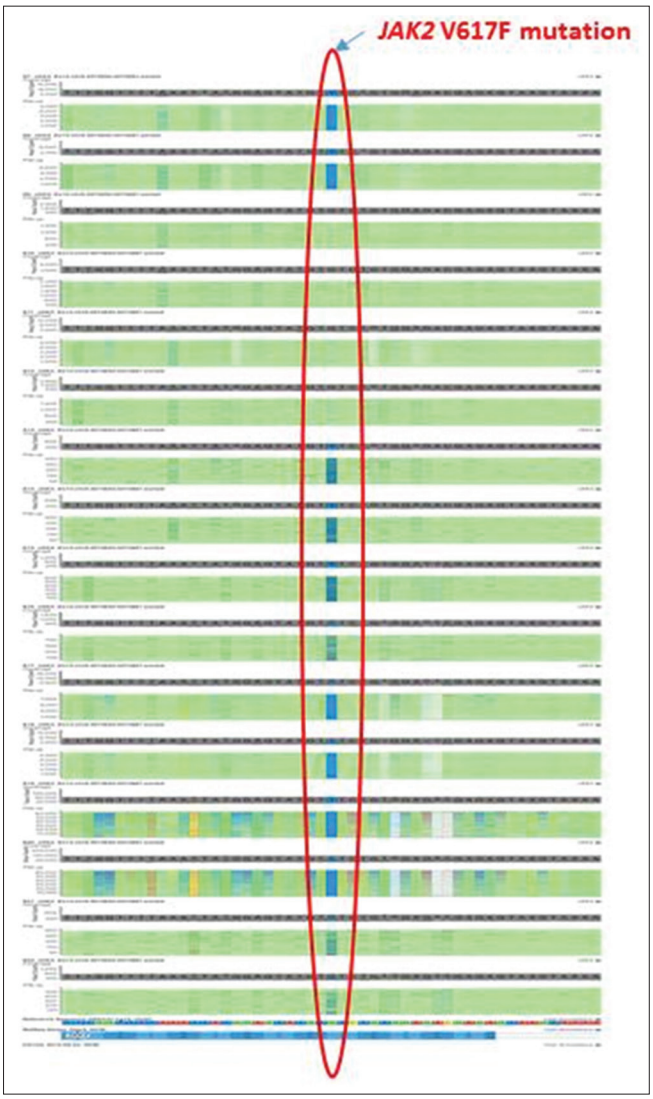

Figure 2: Multiplexed sequencing of 16 samples for *JAK2* exon 14 by next-generation sequencing. The location of the V617F mutation is highlighted. The mutation was detected in 12 of the 16 samples

16 samples). Of the 88 samples tested to date, 63 were positive for Philadelphia chromosome (*BCR-ABL*, t[9;22]), and negative for *JAK2*, *CALR* and *MPL* mutations confirming the diagnosis of CML. These 63 samples were considered as negative controls during the assay development phase. Sequence coverage over the targeted regions can be modulated based on the ratio of PCR product incorporated into the loading mix. While we routinely target coverage of 500X-1000X for each of the amplicons in this assay, we have been able to demonstrate coverage as high as 150,000 reads.

Clinical testing

We tested 25 patients with a clinical diagnosis of Philadelphia-negative CMPN using our custom NGS assay. *JAK2* V617F mutations were detected in 5 patients. No patients had any detectable mutations in exon 10 of the *MPL* gene. Mutations in exon 9 of the *CALR* gene were found in 2 patients. Details of the two cases are presented in Table 1, and sequence data for these two cases are visualized in Figure 3. One was a suspected case of ET and other a suspected case of PMF. A prototypical "TTGTC" Type-II mutation was detected in the ET case at a depth of 27.9% [Figure 3a] which was independently confirmed by bidirectional capillary sequencing. In the other patient, a novel complex mutation was detected. This complex mutation consisted of a 5 bp "TTTGT" frameshift insertion at position c.1153 (one base earlier than the Type-II mutation detected in the other patient) at a relative depth of 13.2% [Figure 3b]. This mutation is classified as a Type-II-like mutation and is specifically annotated as Type 35 (K385fs*47) in the literature.[8,9] In addition, a c.1154A >C transversion (which changes the amino acid from lysine to threonine) at a relative depth of 19%, was also detected [Figure 3b]. Capillary sequencing failed to detect these mutations in the suspected PMF patient, probably due to low level mutation depth.

Discussion

We have developed and validated a multiplexed custom amplicon based NGS assay for Philadelphia negative (*BCR-ABL*/t[9;22]) CMPN targeting exons 12 and 14 of the *JAK2* gene, exon 9 of the *CALR* gene, and exon 10 of the *MPL*

Table 1: Clinical details of the two cases with *CALR* mutation, description of mutations and molecular techniques which confirmed the mutations

Lab id	Age/sex	Clinical diagnosis	CALR mutation	Classification	Molecular technique
16_1973	61 years/ male	Suspected essential thrombocythemia	c.1154_1155 ins TTGTC p.K385fs*47	Type II mutation	Custom NGS assay confirmed by capillary sequencing
16_2156	65 years/ male	Primary myelofibrosis	c.1153_1154insTTTGT K385fs*47 and c.1154A>C	Complex mutation (Type II like and a transversion	Custom NGS assay[#]

[#]Capillary sequencing failed to detect the mutation, probably because of relative low depth of mutation load i.e., 13.2%. NGS: Next-generation sequencing

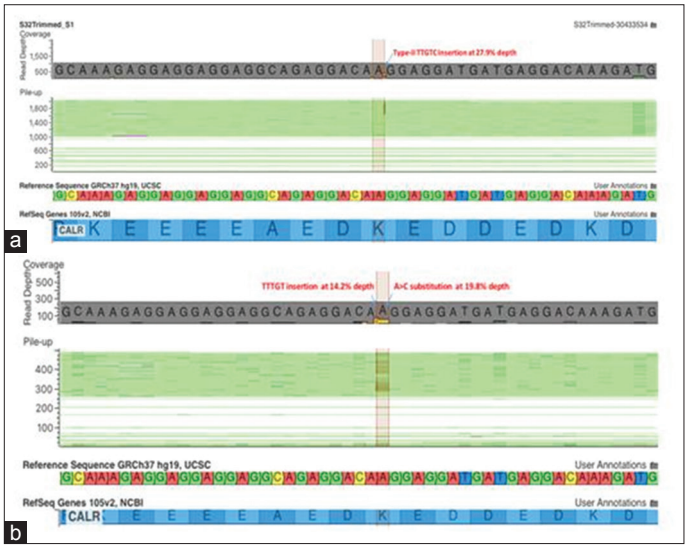

Figure 3: Next generation sequencing data for: (a) The essential thrombocythemia case with a Type-II mutation in exon 9 of *CALR* and (b) the primary myelofibrosis case with a complex Type-II-like mutation along with a transversion in exon 9 of *CALR*

gene. The results generated by the multiplexed NGS assay were confirmed by orthogonal techniques including real-time ARMS PCR and targeted bidirectional capillary sequencing. The positive and negative correlation between the NGS results and the orthogonal technique results was 100% in all samples tested to date indicating the high performance of the assay.

We have currently multiplexed up to 50 CMPN samples in a single NGS run. Theoretically, the number of samples that can be multiplexed is only limited by the number of indices available, the sequencing capacity of the instrument and the physical ability to prepare libraries for sequencing. For other genetic targets, we have currently multiplexed up to 1250 samples in a single NGS run, and envision increasing the multiplexing capacity to 2500 samples in the near future. Such a high degree of multiplexing, coupled with the fact that the entire library preparation protocol and bioinformatics pipeline were developed in-house without the use of expensive kits or software packages, enables a significant reduction of cost of the test when performed at scale.

Another major advantage offered by these kinds of NGS assays is the ability to modulate the depth of sequencing, and thereby, the threshold of detection of mutations in the samples. In a study carried out by Jones *et al.*, the sensitivity of mutation detection by NGS was 1.25%.[10] For this multiplexed assay, we routinely sequence to a depth of ×500–1000. When needed, though, we can also sequence at a depth of ×20,000 or greater which corresponds to a mutation detection sensitivity of 0.5% or lower. While the clinical relevance of such low-level mutations is unclear, it can provide an early indication of emerging mutations during the course of treatment. Finally, because the NGS assay can provide a quantitative result, it may be used for monitoring of minimal residual disease.

We had previously presented a CMPN case with a *CALR* mutation (detected by capillary sequencing), for the first time in India to the best of our knowledge (unpublished data). Here, we report the identification of *CALR* mutations in 2 CMPN patients using our recently developed multiplexed CMPN panel.

The role of *CALR* in CMPN was established fairly recently in 2013 by two independent groups, using NGS-based technologies.[8,9] Klampfl *et al.* carried out exome sequencing of granulocytes and T-lymphocytes, using a paired approach, in six patients with PMF.[9] In another study, Nangalia *et al.* carried out cancer exome sequencing in 151 cases with CMPN.[8] Both groups identified the *CALR* gene as the most frequently mutated gene in Philadelphia-negative and *JAK2* V617F negative CMPN - present at 25–30% of cases with CMPN and 60–80% of cases with *JAK2* V617F negative and *MPL* negative CMPN. All the mutations identified in those studies were frameshift mutations in exon 9 of the *CALR* gene. *CALR* mutations have recently been included in the World Health Organization classification system of CMPN.[5] The *CALR* gene, located on short arm of chromosome 19, contains 9 exons and spans 3.6 Kb [Figure 4a]. The calreticulin protein encoded by this gene is a highly conserved protein that is mainly localized to the endoplasmic reticulum (ER) (OMIM *109091). The calreticulin protein plays a crucial role in calcium homeostasis, protein folding and also in a variety of other cellular processes such as proliferation, steroid sensitive gene expression, cell adhesion, apoptotic cell clearance, cell migration, and immunomodulation. Exon 9 of the *CALR* gene codes for the C-terminal domain of the calreticulin protein. Pathogenic mutations in this region produce a mutant protein with an altered C-terminus missing the acidic domain. This domain contains the "Lys-Asp-Glu-Leu (KDEL)" signal comprising the amino acids KDEL, and is required for appropriate subcellular localization/retention inside ER of the calreticulin protein [Figure 4b]. More than 50 mutations in *CALR* gene have been identified to date.[5,8,9] Except for a few rare nonrecurrent mutations, most of the reported mutations are insertions/deletions (InDels), which disrupt the reading frame in exon 9 of the *CALR* gene. Klampfl *et al.*

have classified the InDels in the *CALR* gene into two main categories - Type-I (52 bp deletion) and Type-II (5 bp "TTGTC" insertion) [Figure 4a]. In their patient cohort, these two mutation types were present in 80% of cases with *CALR* gene mutations. They also observed that in cases of PMF, Type-I mutations are more common.[9] Subsequently, these mutations have been categorized as Type-I/Type-I-like and Type-II/Type-II-like mutations (with the originally described Type I/II mutations serving as prototypes) on the basis of the α-helical propensity of 31 amino acids located at the C-terminal of mutant calreticulin protein.[11] The α-helix forming propensity is lowermost in Type-I/I-like mutations and similar to or greater than that of the wild type of protein in Type-II/II-like mutations.

Conventional techniques to identify mutations in the *CALR* gene mutation include capillary sequencing, high-resolution melt analysis (HRMA) and quantitative reverse transcriptase PCR (qRT-PCR).[6,10] Small InDels, which cause frameshift mutations in the *CALR* gene, present at somatic mutation loads of 35–50% can be routinely detected by capillary sequencing, which typically has a detection sensitivity of 15–20%.[6] The technique may, however, miss large deletions/duplications, other copy number variations, and lower level mutations. Furthermore, the method is time-consuming, labor intensive and unamenable to a high degree of multiplexing. The detection sensitivity of HRMA, which can detect mutations down to 3–5%, is higher than that possible through capillary sequencing.[6,10] While HRMA is capable of screening the two most common types of *CALR* mutations efficiently, it can produce indeterminate results for other InDels or point mutations. The results of HRMA usually need to be confirmed by other molecular techniques. The qRT-PCR method is considered reliable for the detection of the two most Type-I/II mutations but is not readily amenable to interrogate unknown/novel mutations due to the need to know the exact mutation *a priori*. In contrast to conventional techniques, NGS methods can identify all types of mutations (point mutation, InDels, and copy number variations) in a single run along with quantification of mutation load and the ability to multiplex many samples. Jones *et al*. performed a study comparing the detection sensitivity of *CALR* gene mutations using four techniques - capillary sequencing, HRMA, qRT-PCR and NGS.[10] They concluded that NGS is the most sensitive approach and is capable of detecting mutations down to a level of 1.25%. Most NGS assays for targets like *JAK2*, *CALR,* and *MPL*, however, are part of much larger gene panels. To the best of our knowledge, there are limited reports of small targeted multiplexed panels incorporating these genes.

In the current CMPN case examples, we have detected a Type-II and a complex mutation (Type-II-like with a nearby transversion) in exon 9 of the *CALR* gene using our targeted NGS assay. Patients with *CALR* mutations, in general, have a lower risk of thrombosis, higher platelet count, lower white blood count and overall higher survival rates than *JAK2* V617F positive patients. PMF patients with *CALR* gene mutations tend

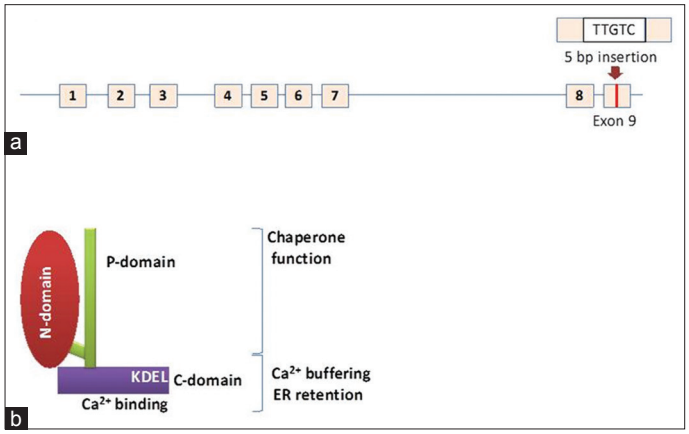

Figure 4: (a) Diagrammatic representation of *CALR* gene showing the location of the detected mutations in exon 9 and (b) diagrammatic representation of the calreticulin protein. Loss of the Lys-Asp-Glu-Leu signal prevents retention in the endoplasmic reticulum. The mutated *CALR* protein can fuse with the cytokine thrombopoietin receptor, which acts through the JAK-STAT pathway keeping hematopoietic cells immortal

to have higher hemoglobin levels compared to PMF patients with *JAK2* mutations.[5,11] In a study by Pietra *et al.*, Type-II-like mutations were found to be more common in ET patients (39% prevalence) as compared to PMF patients (15%).[12] They also noted that there were no differences in clinical presentations between patients with Type-I-like mutation and those with Type-II-like *CALR* mutations. Overall survival, however, was better for patients with *CALR* Type-I-like mutations as compared with those carrying the *JAK2* V617F mutation and was similar between patients with *CALR* Type-I and -II-like mutation. PMF patients with Type-II-like mutations had milder clinical courses and lower risks of thrombosis and myelofibrotic transformations than those with *JAK2* or *CALR* Type-I like mutations.[12]

The curative treatment for PMF remains allogeneic stem cell transplantation in a defined subset of patients. Decisions regarding stem cell transplantation depend on the age of the patient, risk category, percentage of blasts in peripheral blood, and frequency of blood transfusions.[5] Recent experiments suggest that cells expressing mutant *CALR* have cytokine-independent growth of cells involving the JAK-STAT signaling pathway and have been shown to respond to *JAK2* inhibitors.[13,14] Ruxolitinib is the only *JAK2* inhibitor to have been approved and is being adopted into clinical practice. Other JAK inhibitors such as fedratinib, momelotinib, and pacritinib have entered into Phase III clinical trials.[5] In a study by Cazzola and Kralovics in two *CALR* mutation positive patients with the JAK inhibitor fedratinib, the authors noticed a favorable response in terms of symptom relief and a 50% reduction in spleen size.[14] A few other studies have shown that *CALR* gene mutations also downregulate the calcineurin-nuclear factor of activated T-cells signaling pathway.[15] This pathway is a negative regulator of megakaryocytic and myeloid lineage cells in hematopoietic stem cells. This could explain the propensity for *CALR* gene mutations in ET and PMF patients but not in PV, and opens up the potential of treatment strategies for ET and PMF through inhibition of these pathways.

Our custom NGS assay serves as a template for the addition of other genes that have recently been implicated in CMPN. These include *ASXL1, TET2, CBL, DNMT3A, CEBPA, SH2B3, SF3B1, TP53, SF3B1,* and *U2AF1*.[5] The use of these genes will likely improve diagnostic efficiency in CMPN patients and hence the development of a single comprehensive and cost-effective testing for all cases with CMPN. This, in turn, will provide the hematologist with the wider options for targeted therapies in the future.

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