

Research paper

Molecular stratifications of Myeloproliferative Neoplasms according to WHO 2008 classification for hematopoietic and lymphoid tissues (experience from Qatar).

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ABSTRACT

Major changes occur in the classification of MyeloproliferativeNeoplasms (MPNs) since 2008 when WHO introduced its new classifications for hematopoitic and lymphoid tissues where the nomenclature changed to Neoplasm ,JAK 2 and other clonal abnormalities included in diagnosis of Polycythemia Vera (PV) ,Essential Thrombocytemia(ET) and Primary Myelofibrosis(PMF),the threshold for platelets and sustainability redefined as during the period of work up very little is known about Myeloproliferative Neoplasms among Arab Populations this project is highlighting and describing the MPNs in this part of the world with special focusing for Arab population and with special interest to study in depth ` the familial myeloproliferativeneoplasm'samong Arab populations.

Introduction

Myeloproliferative disorders (MPDs) are hematological malignancies characterized by an accumulation of mature cells in the peripheral blood(1). Usually, Chronic Myeloid Leukemia (CML), a well characterized entity harbouring the recurrent t(9;22) translocation and the resulting BCRABL1 fusion gene, is separated from the classical MPNs such as Polycythemia Vera (PV), Essential Thrombocytemia (ET) and Primary Myelofibrosis (PMF), in which a molecular abnormality has long time been ignored. In the latter group, the recurrent V617F mutation in the exon 14 of the JAK2 gene has been identified in 2005 and is currently a key marker for MPNs diagnosis as this mutation is present in 90%, 60% and 50% of PV, TE and PMF respectively .In 2007, (2)novel recurrent mutations clustered in a highly conserved region in exon 12 of the JAK2 gene have been described in patients with PV or Idiopathic erythrocytosis. Exon 14 and exon 12 mutations differ by 2 main characteristics: the V617F mutation is limited to only one base change (G1849T) found in all subtypes of MPNs as well as in splanchnic vein thrombosis and some myelodysplastic syndromes patients .Exon 12 mutations on the other hands are extremely variable in sequence and so far restricted to polycythaemia patients. In a recent study, using allele specific PCR,(3) we reported the presence of JAK2 exon 12 mutations in 8 out of 24 PV patients negative for JAK2 V617F mutation, but failed to detect these mutations in patients with idiopathic erythrocytosis. The detection of JAK2 exon 12 mutations is technically much more complicated than V617F mutation detection. Although some mutants are more frequent than others there has been an increasing number of different deletions, insertions or base changes described in the literature since the initial description.(4)

Until recently, mutant detection had to be addressed either by direct sequencing (of low sensitivity) or allele specific PCR (of good sensitivity) but inadequate in a routine diagnosis setting with so many different mutations requiring multiple individual PCR reactions. High Resolution DNA Melting curve analysis (HRM) is based on DNA melting in the presence of saturating DNA binding dyes. Sequence variants are inferred from changes in the melting transition of the PCR product as, depending on their GC content, length or sequence, different PCR products have different melting temperatures, whether mutated sequences are known or not. HRM methods have now been adapted to real-time PCR instruments(5) and, compared to sequencing or AS-PCR, represent high throughput and time saving methods with the further advantage of reducing post-PCR handling of PCR products. HRM technology has been adapted to the identification of bacterial species or subtypes, human SNP genotyping or mutation detection. However, instruments vary widely in their ability to genotype variants by whole amplicon melting analysis. Similarly, several DNA binding dyes may be used with variable success. Because HRM technology could be a rapid and convenient tool for detecting the various JAK2 exon12 mutations, we decided to develop one such method with the prerequisite that it should be reliable enough to give similar results on 2 different instruments in 2 different sites. Then, the assay has been further validated on a cohort of 8 different mutants and 4 non mutated DNA in 2 additional centers, of whom one functioned in a blind manner.

Patient and methods

Between May 2012 and Dec 2014, suspected MPNs patients aged 18- 70 years recruited at National center for Cancer and Research (NCCCR), Hamad Medical Corporation (HMC) according to WHO 2008 criteria

All procedures were performed in accordance with the Helsinki Declaration and approved by the Hamad Medical Corporation research committe.

Peripheral blood and bone marrow samples

3- 5 ml of Peripheral blood(PB) and / or Bone Marrow marrow (BM) samplesin EDTA were collected every 3 months and bone samples were collected every 6 months

DNA extraction and quantification

Patients genomic DNA was extracted from whole blood using Qiagen and Promega DNA extraction procedures according to the manufacturer's instructions The concentration and purity of RNA were measured by the nanodrop machine at optical density (OD) 260/280nmand DNA samples were stored in -20ºC freezers

Sample Size

The numbers of samples which collected from suspected MPN's at 1-36 months of the study are around 3000 samples Sampling Technique: 3- 5 ml of Peripheral blood in EDTA and / or bone marrow samples will be collected from suspected MPN's cases Study Subjects.

Recruitment of patients

Suspected 450 MPNs Adult patients 18- 70 years have been recruited at National center for Cancer and Research (NCCCR), Hamad Medical Corporation (HMC) according to the following (inclusion / exclusion) criteria

Polycythemia Vera (PV)

Inclusion criteria 1.Hemoglobin > 18.5 g/dL in men, 16.5 g/dL in women or other evidence of increased red cell volume 2.presence of JAK 2v617f or other clonal abnormalities 3.Serum erythropoietin: normal or low. 4.Bone marrow biopsy showing hypercellularity for age with trilineage growth (panmyelosis) with prominent erythroid, granulocytic, and megakaryocytic proliferation Exclusion criteria: 5.High eryhropiotein Level.

For Essential thrombocythemia (ET) AND Primary myelofibrosis WHO 2008 criteria were used as inclusion criteria

Exclusion criteria:

1. not willing to participte in the study

2. not fulfilling WHO criteria for Myelofibrosis

3.age less than 18 or more than 70

JAK2 Mutation screening and quantification

Suspected MPNs cases were screened and quantified for JAK2 V617F using JAK2 MutaScreenand JAk2Muta Quanta Kits (Qiagen)

This multiplex assay is based on two probes (double-dye oligonucleotide labeled with a 5' reporter dye and a downstream, 3' quencher dye with hydrolysis principle). The two probes designed to detect and quantify JAK J617F and WT.

During PCR, forward and reverse specific primers and two probes for JAK J617F and WT hybridize and amplify the region of interest. The hydrolysis probes exploit the 5' →3' exonuclease activity of the Thermusaquaticus (Taq) DNA polymerase. When the probes are intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förster-type energy transfer. If the target of interest is present, the probes specifically hybridize /anneal to its specific target between the forward and reverse primer sites and is cleaved by the 5'→3' exonuclease activity of the (Taq) DNA polymerase .The probe fragments are then displaced from the target, and florescence signal is released and detected. The increase in fluorescence signal is detected only if the target sequence is complementary to the probe.

Standard Curves (SCs)

Six serial dilutions (5 $x10^1$, 5 $x10^2$, 5 $x10^3$, 5 $x10^4$, copies /5µls) of JAK J617F and WT and plasmids were used to establish/construct two standard curves as well as to determine the concentration of JAK J617F type and WT in patients samples.

The JAK2 V617F Positive Control (DNA 100% V617F), V617F Negative Control (DNA 100% WT), and Reference Sample (DNA 2% V617F) were used.Results interpretation If the ratio of FAM/VIC for the patient sample is more or equal to the ratio of reference sample(DNA 2% V617F), the result is positive and patient has the V617F mutation and if the ratio of FAM/VICfor the patient samples is less than reference sample the result is negative and patient doesn't have JAK2 V617F mutation

DNA extraction

Patients genomic DNA was extracted from neutrophilis using QIAamp DNA extraction procedures, and DNA samples will be stored in -20ºC freezers located in Al- Amal hospitalJAK2 Mutation Screen allelic discrimination assay would be used: Briefly, Genomic DNA will be extracted from whole blood or bone marrow and an allelic discrimination assay with two TaqMan probes will be used (multiplexed assay). One is match to the allele 1 sequence (eg. the wildtype allele), the other one is match to the allele 2 (eg. the allele with a mutation). Each probe is labelled with a distinctive fluorescent dye at its 5' end (Reporter) such as FAM or VIC, and contains a nonfluorescent Quencher at the 3' end. The probes also contain a minor grove binder (MGB) permitting the use of shorter probes with greater stability and thereby a more accurate allelic discrimination. During the extension phase of the PCR, the perfectly matched probe is cleaved by the 5'→3' exonuclease activity of Taq polymerase, separating the Reporter dye from the Quencher and thus releasing detectable fluorescence. The mismatched probe will be displaced rather than cleaved by the Taq Polymerase and no reporter dye is released. The fluorescence signal (FAM or

VIC) generated is collected at the end of the PCR (endpoint) and immediately indicates the presence of the targeted sequence(s) in the sample (wildtype allele, mutated allele or both) in each experiments. V617F Positive Control (DNA 100% V617F), V617F Negative Control (DNA 100% WT), and Reference Sample (DNA 2% V617F) will be used (figure 2) Results interpretation If the ratio of FAMTM/VIC® for the patient sample is more or equal to the ratio of reference sample(DNA 2% V617F), the result is positive and patient has the V617F mutation and if the ratio of FAMTM/VIC® for the patient samples is less than reference sample the result is negative and patient doesn't have V617F mutation

JAK2 exon 12 to 15 mutations screening

The negative MPNs patients for JAK2V617F mutation was screened to JAK2 exons 12-15

RNA extraction and quantification

RNA was extracted from 1× $10^7$of white blood cells in guanidiniumthiocyanate buffer (GTC) combined with ion-exchange chromatography using spin-columns (QIAamp RNA blood Mini kit, Qiagen) according to the manufacturer's instructions.The concentration and purity of RNA were measured by the nanodrop machine at optical density (OD) 260/280 nm.

cDNA synthesis:

cDNA was synthesized from 1µg of purified RNA using reverse transcriptase RT-Dx kit (Ipsogen) according to the manufacturer's instructions.

High-Resolution Melting (HRM) Curve Analysis

Amplification reactions and HRM was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) using default parameters. Normalized melt curves and difference plots were analyzed using HRM Software version 2.0 (Applied Biosystems).

Sanger Sequencing

The suspected cases identified by HRM were further investigated via sanger sequencing to confirm mutations. HRM product(8) was treated with ExoSAP or Qiagen PCR purification Kit, and then the purified PCR product was used as template for bidirectional sequencing using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA) on the ABI 3730 Genetic Analyzer. Sequences are aligned to wild type reference sequence and assessed for the presence of mutations.

JAK2 (V617F) negative PV patients would be screened for JAK2 (exon12) mutations using HRM JAK2 exon 12 with direct sequencing. High resolution melting curve assay Jak2 exon 12 will be amplified and HRM assay will be conducted to distinguish between the various JAK2 exon 12 mutated alleles. Sequencing of HRM products HRM product will be treated with ExoSAPIT or Qiagen PCR purification Kit, and then the purified PCR product will be used as template for sequencing with the Big Dye Terminator kit.Peripheral blood and bone marrow will be collected & DNA will be extracted from suspected ET patients as mentioned on (1.4.1 & 1.4.2)

Suspected ET patients will be screened for JAK2 (V617F) using RQ – PCR

JAK2 (V617F) mutation as mentioned in section (1.4.3)1.5.2 JAK2 (V617F) negative ET patients would be screened for MPL (W515L/K) mutations using RQ-PCR MPL.

MPL Mutations Screen allelic discrimination assay

In an allelic discrimination assay, two different probes will be used (multiplexed assay). One is a perfect match to the the wild type allele sequence (eg. the wild type allele), the other one is a perfect match to the mutation allele sequence Each probe is labelled with a distinctive fluorescent dye at its 5' end (Reporter) such as FAM or VIC, and contains a nonfluorescent Quencher at the 3' end. The probes also contain a minor grove binder (MGB) permitting the use of shorter probes with greater stability and thereby a more accurate allelic discrimination. During the extension phase of the PCR, the perfectly matched probe is cleaved by the 5'-3' exonuclease activity of Taq polymerase, separating the Reporter dye from the Quencher and thus releasing detectable fluorescence. The not matched probe will be displaced rather than cleaved by the Taq Polymerase and no reporter dye is released. The fluorescence signal (VIC or FAM) generated is collected at the end of the PCR (endpoint) and immediately indicates the presence of the targeted sequence(s) in the sample (wild type allele, mutated allele or both) (figure 4) 1.5.2.1 Results interpretation: If the ratio of FAMTM/VIC® patient result is more or equal of ratio of cutoff sample (COS 1.5% of WL/K), result is positive and patient has the WL or WK mutation and if the ratio of FAMTM/VIC® for the patient samples is less than result of COS the result is negative and patient doesn't have WL or WK mutations

HRM method Three instruments were used in 4 different French centres: a Light Cycler 480 (Roche Applied Sciences) in Toulouse and Brest, an ABI 7500 fast (AppliedBiosystems) in Paris and an ABI 7900 (AppliedBiosystems) in Creteil. PCR reactions were performed in a 12µl final volume containing 20 ng of genomic DNA and 0.2µM of forward (5'- ACCAACCTCACCAACATTACAGAG-3') and reverse (5'- AAAAGGACAAAAAAGACAGTAATGAGTATC-3') primers defining a 184 bp amplicon. When LightCycler apparatus was used, the LC480 HRM master mix (Roche), containing Resolightc as DNA binding dye, was used and 3mM MgCl2 was added, whereas the AmpliTaq Gold PCR Master Mix (AppliedBiosystems) with 1.5 µM of Syto-9 (Invitrogen) were used with the ABI 7500 fast or ABI 7900 instruments. Amplification was performed by 50 cycles of 95°C for 15 secs, 63°C for 15 secs and 72°C for 25 secs followed by a melt according to each manufacturer instructions. Sequencing analysis PCR reactions were performed in a 20 µL reaction volume containing the following: 10 pmol of each primer

forward primer was 5'-CTCCTCTTTGGAGCAATTCA-3' and reverse primer was 5'- GAGAACTTGGGAGTTGCGATA-3', 1x PCR buffer (Qiagen), 200 µM each dNTPS (Invitrogen), 1U of HotStarTaq DNA polymerase (Qiagen) and 20ng of DNA. Cycling conditions were as follows: 95°C for 15 min, 35 cycles of 94°C for 20 s, 59°C for 20 sec, 72°C for 45s followed by a final elongation step at 72°C for 10 min. PCR Amplified fragment was 495 bp in length. Sequencing analyses were performed using a fluorescent-tagged dideoxy chain termination method with a 3130XL-DNA sequencing system (Applied Biosystem).

HRM analysis is a suitable method for JAK2 exon 12 mutations detection We previously reported several JAK2 V617F negative polycythemia patients with JAK2 exon 12 mutations detected by allele specific Polymerase Chain Reaction (AS-PCR(10). In the present study we first used total blood DNA from these positive patients in order to set up a new assay using HRM technology in 2 centres. The assay was first developed on a Lightcycler 480 (Roche) in Toulouse, and then tested on an ABI 7500 fast (AppliedBiosystems) instrument in Paris. Using HRM method allowed to identify every patient known to be mutated, whereas all control patients showed a wild type profile. These results confirmed that High Resolution DNA Melting curve analysis is a suitable method for the detection of JAK2 exon 12 abnormalities. In order to test for the reproducibility of the method we have analysed 2 positive samples characterized by different mutant sequences in 3 independent experiments, each performed at 1 week interval. Results were highly similar in all three experiments (not shown), proving that the HRM method tested herein could have sufficient robustness for a diagnostic purpose. Though it is now widely admitted that JAK2 V617F mutations can be detected with similar efficacy in DNA from peripheral blood or purified granulocytes, this has not been extensively studied for the JAK2 exon 12 mutations. We compared in 2 patients the results obtained with DNA extracted from total blood or from purified granulocytes. Similar results were observed whatever the source of DNA Applications of the HRM analysis Samples (n=39) of patients with either idiopathic erythrocytosis or PV from our previous report which were negative by AS-PCR for exon 12 mutations were analysed by the HRM method and were again found negative. In the analysis of new patients addressed to our laboratories for MPD diagnosis, 9 out of 35 patients presenting with an increased hematocrit, low Epo levels and absence of the JAK2 V617F mutation, harboured a mutation confirmed by sequencing analysis. Validation of the method in 2 additional centres. It has been reported that important discrepancies could be observed when one HRM method was used on different instruments (9). In a preliminary approach, we thus compared the diagnostic accuracy of our method by analysing the 9 positive samples on 2 instruments, in 2 separate laboratories in Toulouse and Paris. Each laboratory used different DNA binding dyes (Resolightc on the LC480 instrument and Syto-9 on the ABI 7500 instrument) also reported to be source for discrepancies. Although shifting temperatures and curves shapes were somewhat different between the two instruments, results were undoubtedly similar in their interpretation. The validation of the method was completed by analysing 8 samples bearing different mutated sequences (clinical data and mutant subtypes are given in Table 1) in 2 additional laboratories, Brest and Creteil, which had not participated in the set up of the HRM assay. In one of the two labs (Creteil), the analysis was performed in a completely blind manner, on12 anonymous DNA samples (8 mutated and 4 wild type) on an ABI 7900 instrument (Applied Biosystems) whereas a LC480 instrument (Roche) was used in Brest. The HRM method was equal. **(Figure 1), (Figure 2), (Figure3).**

Results and Discussion

Results

450 patients were classified into PV, ET & PM. Out of 180 PV, 95% of cases were positive for the JAK2 V617F & 5% of cases were negative for other mutations. Out of 240 ET, 49% of cases were positive for JAK2 V617F, one had MPL S505N mutation & 50% of cases were negative for other mutations. Out of 30 PMF, 35% of cases were positive for JAK2 V617F & 1 unclassified case was characterized by DVT had JAK2 exon 13 mutation (R564L). 11 samples were successfully sequenced, with a mean depth of 1500 reads & the FASTQC plugin indicated good quality sequencing metrics. JAK2 V617F, JAK2 exon 12-15 & MPL (S505N, W515 L/K) negative samples tested before via RQ-PCR, HRM & sequencing were called negative by NGS. NGS identified novel deleterious mutations in MPNs patients. Out of 6 familial cases, 5 patients (P1- P5) were ET & 1 patient (P6) was PV. P1 had JAK2 V617F, ASXL1 T600P, CBFB G180S, THPO S184R & ITGA2 R76Q, P2 had JAK2 V617F, MPL A554G & ATM F582L, the other three Patients (P3, P4 & P5) had CLAR K385fs*47 & one PV patient (P6) had TYK2 E1163G, ASXL1 P808H, PDGFRB P4L & TERT G300fs. Among the patients & healthy individuals, mutations/SNVs such as MPL P106L, K553N, SH2B3 L476F, ATM F1036F KIT N564S & TET2 T730R were also found. A complex combination of mutations in JAK2, THPO, ITGA2 & MPL genes occurred in ET patients & coexistence of several oncogenic events in TYK2, ASXL1, PDGFRB & TERT occurred in PV patient. This finding may also suggest that the MPNs phenotype may depend on presence of other mutations. It is worth mentioning that the presence of ATM variant in P2 is associated with increased risk of CLL. Somatic CALR type-2 mutation was identified in 3 ET (non mutated JAK2 or MPL) patients. This mutation is 5-bp TTGTC insertion in exon 9 that generates a mutant protein with a novel C-terminal (p.K385fs*47). In patients & healthy individuals, a heterozygous germ-line mutation in exon 3 of the MPL gene (MPL P106L) has been observed.

Conclusion

Myelooproliferative Neoplasms are prevalent among Arab Populations with more specific feature of presence of familial cases as well as predilection to affect younger age groups which requires further investigations by Whole exome sequencing or whole genome sequencing to further explore the disease in this unique category of patients.

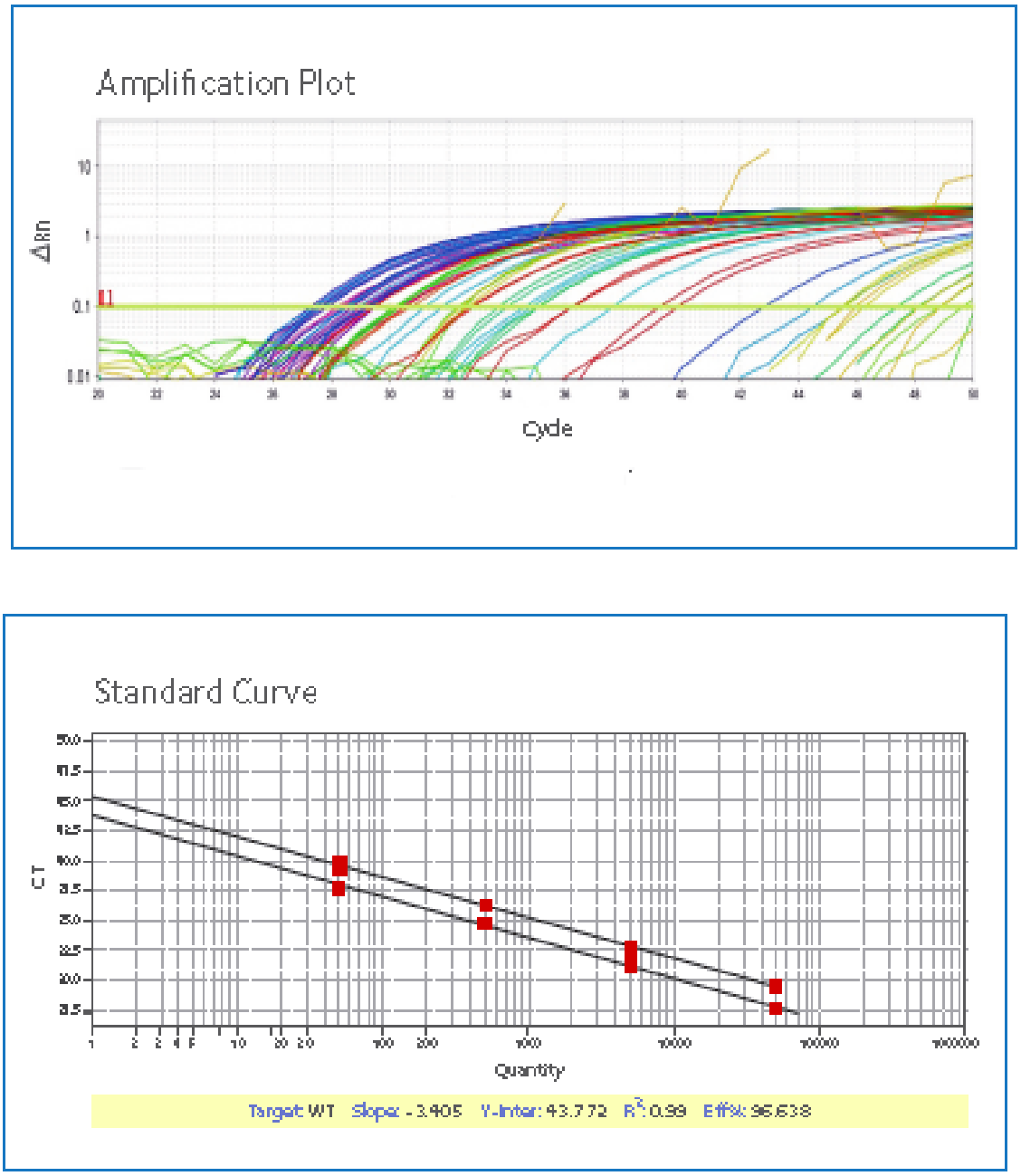

Figure 1 During PCR, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites. The 5'à3' exonuclease activity of the DNA polymerase cleaves the probe between the reporter and the quencher and the fluorescence is released. This technology called quantitative allele discrimination which allows a sensitive and accurate detection of SNPs. Serial dilutions of VF and WT plasmids were used to construct the standards curves 50 , 500 , 5000, 50,000 copies for both WT and VF. These are the Standard curves for JAK2 V617F and WT that used to calculate and quantify of unknown samples.

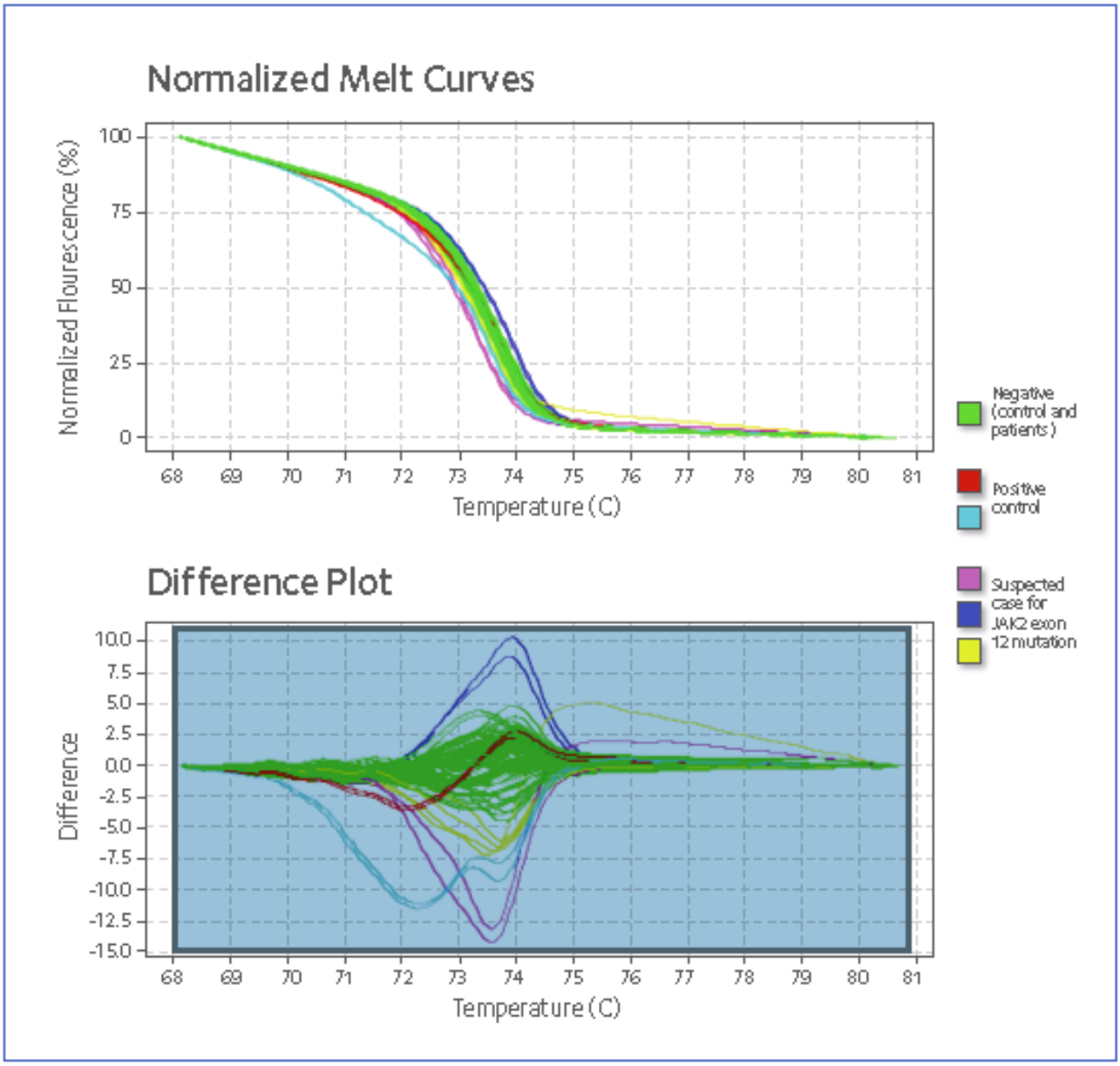

Figure 2:MPNs cases negative for jak2 v617f were studied by HRM which is screening methodology to detect genetic mutation/SNPs in Jak2 exon 12 -15. The principal of the procedure: That after amplification of region of interest in presence of binding dye for ds DNA, amplified target is gradually denatured by increasing the temperature, releasing the dye and results in a drop in fluorescence and allow the detection of a single base change between identical nucleotide sequences.

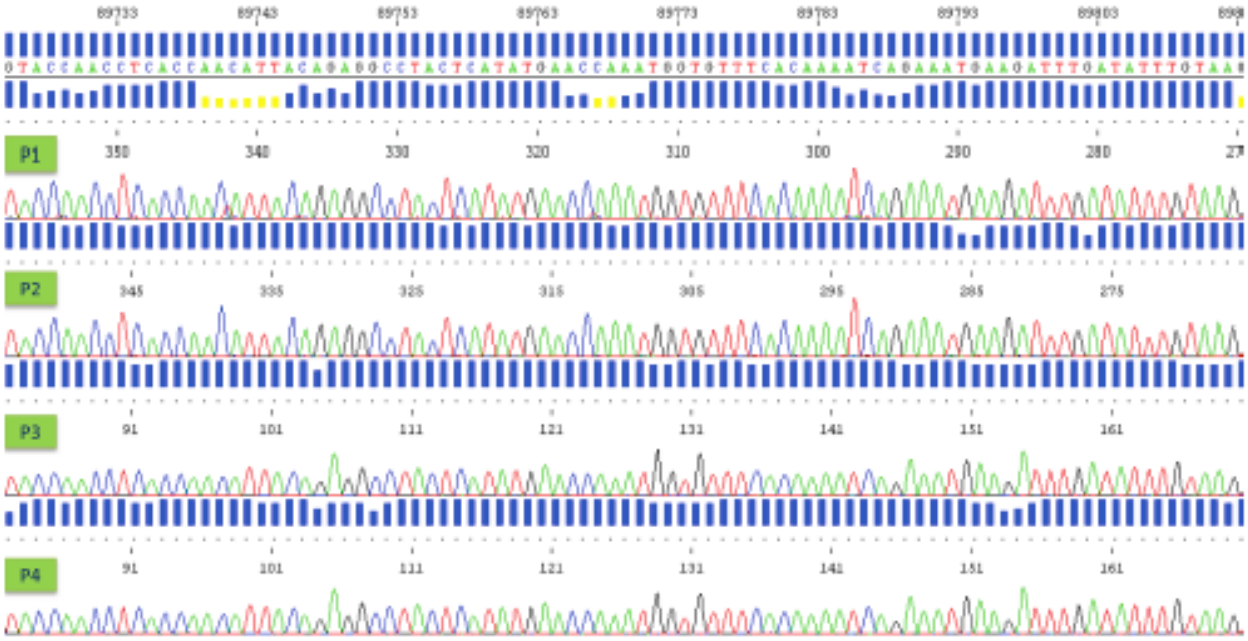

Figure 3: The suspected cases identified by HRM were further investigated via sanger sequencing to confirm mutations.

Conflict of Interest

This research was conducted as part of the Qatar national Research Fund-sponsored project "novel approach in Molecular pathophysiology of Myeloproliferative neoplasms: What determines phenotypes of JaK2 Mutations (Qatari prospective)" (npRp number 4–471–3–148). this is applicable to Yassin Ma and al-dewik n.

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