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Screening for a Single *JAK2*(p.V617F)Mutation Allele in Suspected Patients with Myeloproliferative Neoplasms (MPN) at HIWA Hospital

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Abstract

Detection and diagnosis of the myeloproliferative neoplasms (MPNs) are difficult to predict without screening for genetic causes, includingpolycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF) and chronic myeloid leukemia (CML). A novel mutation in the Janus kinase 2 (*JAK2*) gene has been described as a genetic marker prime for all four-types of MPNs.The specific c.1851G>T (p.V617F) mutation leads to constitutively activetyrosine kinase activity of JAK2, inducing downstream JAK/STAT pathways of cytokine signaling. The existence of the *JAK2*(c.1851G>T; p.V617F) mutation has a clinical importance in diagnosis of MPNs. In this study, amplification refractory mutation system (ARMS) assay for a specific mutation detection was utilized, which is an Allele Specific Oligonucleotide (ASO) based multiplex PCR method. Genomic DNA samples were isolated from total blood samples from suspected MPN patients, who visited Hiwa hospital (the main teaching hospital for cancer in Sulaimani city). The aim of this study was to screen for *JAK2* mutation c.1851G>T (p.V617F) on a group of suspected patients for MPNs (100 patients). The result shows that 65% of patients had indeed c.1851G>T mutation and the rest 35% of patients were normal for the mutation. In this study, we have shown the ARMS assay method to be quick, simple, cheap, reliable, and gives sufficient sensitivity for positive detection compatible forclinical diagnostic purposes. Therefore, the assay can be used for early diagnosis of MPNsin diagnostic laboratories with limited resources, such as in our health care system in our locality.

Introduction

The Janus kinase (JAK) proteins families are family of intracellular non-receptor tyrosine non-receptor tyrosine kinases mediating intracellular cytokine signaling through STAT (Signal Transducers and Activators of Transcription) signal transduction. Molecular genetic screening have found somatic *JAK2* c.1851G>T (p.V617F) mutations that activate constitutively the JAK-STAT signaling in almost all patients with myeloproliferative neoplasms (MPNs) (LaFave and Levine, 2012).

Majority of Chronic myeloproliferative neoplasms are negative for chromosome 22 shortening, which are grouped in three different human diseases: polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). These abnormal conditions are defined as clonal diseases originated from the

stem cell proliferation derivedfrom one or more of the three hematopoietic bone marrow linages (granulocytic, erythroid or megakaryocytic) (Tefferi and Gilliland, 2005).

The mutation of the *JAK2* gene c.1851 G>T (p.V617F) was first discovered in 2005, where JAK2 converted to gain-of-function mutation, caused by a single nucleotide change G into T at position 1851 in the exon 14, resulting in alteration of the valine codon into phenylalanine at position 617 (*JAK2* c.1851 G>T ; p.V617F), which is located in the pseudo-kinase domain (JH2) of the JAK2 protein. The cytogenetic location of*JAK2* geneon the short p-arm of chromosome 9, band 9p24.1 is mapped by *in situ* hybridization. More precisely, the complete gene sequence spans between 4,985,244 bp to 5,128,182 bp(Baxter *et al*., 2005;James *et al*., 2005;Levine *et al*., 2005). The somatic *JAK2* c.1851 G>T (p.V617F) mutation is positively detected in 95% of the patients with PV and in 50% of ET and PMF patients, respectively(Vakil and Tefferi, 2011).

The mutated *JAK2* gene has an active tyrosine kinase in a constitutive manner and confers the ability to induce proliferation signal autonomously in hematopoietic cellular lineages, supporting the formation of elevated erythroid pool independently of erythropoietin (Baxter *et al*., 2005).

The most obvious clinical hallmarks of PV are the increased red cell mass and hematocrit, while ET is characterized by elevated platelet counts and elevated megakaryocyte proliferation in the bone marrow. The main clinical feature of PMF are knownas anemia, splenomegaly, leukocytosis, and other intrinsic symptoms. On-the-other-hands, patients who are diagnosed with PV, ET and PMF are atelevated risk of thrombosis and distorted bleeding (LaFave and Levine, 2012).

The aim of this study is to detect a specific*JAK2* missense mutation c.1851G>T (p.V617F) and determine the prevalence of the mutation in patients with PV, ET and PMF. In addition, the clinical manifestation will be examined at individual phenotype level, regarding the impact of *JAK2* c.1851G>T (p.V617F) mutation.

Materials and Methods

The experimental project plan was approved by ethical committee in the faculty of medicine at the University of Sulaimani. The main common diagnostic criteria in this study are based on WHO diagnostic criteria in 2008, as following: ***Polycythemia vera (PV);***Hemoglobin level > 18.5 g/dL in men, 16.5 g/dL in women. Other features were recorded, such as an increased red cell mass, splenomegaly, or presence of *JAK2* c.1851G>T (p.V617F) mutation. or other functionally similar mutation.***Essential thrombocythemia (ET);***the sustained high platelet counts are ≥ 450 000/ μL, and also to demonstrate *JAK2* c.1851G>T (p.V617F) mutation by semi-quantitative conventional polymerase chain reaction (cPCR) or quantitative real-time PCR (qPCR). ***Primary myelofibrosis (PMF);***the existence of megakaryocyte proliferation and atypia (structural abnormality inside cells), presented by either reticulin or collagen fibrosis in the bone marrow, increased granulocyte proliferation and decreased erythropoiesis, splenomegaly or demonstration of *JAK2* c.1851G>T (p.V617F) mutation by cPCR or qPCR.

Sample collection: Two ml of venous blood was collected in EDTA tubes from 100 patients after taking the informed consent for this study that was clinically suspected as MPN according to hematologist's clinical diagnosis atHiwa hospital, Ministry of Health, Kurdistan Governmental Region, Iraq. These patients show an elevated hematocrit for PV but for other MPNs were decreased hematocrit level with abnormal blood film finding, and also most of the patients have had splenomegaly phenotype whose ages ranged 19-81 years old. Blood sampleswere transported in a cool box to laboratory and total genome was extracted within 2 hours from taking the samples.

Genomic DNA extraction: Genomic DNA exactions were performed using Geneaid DNA extraction kit (New Taipei City - Taiwan).

PCR amplification: The DNA region from *JAK2* gene was amplified by PCR using specific oligonucleotide primers (Bioneer-South Korea). All were tested for the *JAK2* c.1851G>T (p.V617F) mutation using a multiplex PCR as shown in Table 1.

The PCR amplification include initial denaturation for 4 min. at 94°C, then 35 cycles: each cycle consists of 94°C for 30 sec., 56°C for 30 sec., and 72°C for 35 sec. (according to primer manufacturer) and final

extension 72°C for 7 minutes. The PCR amplification products (amplicons) were analyzed on 2% of agarose gels at 90 V, 85 mA, 9 W for 90 min.

Table 1: The PCR reaction contents of primer, DNA, master mix. The final volume of each PCR reaction was 25 was 25 µl.

Components	Description	Volume
Template	DNA	2 µl
Multiplex Primers (10 µM Solution)	FI – 1 µl RI – 1 µl Fn – 0.5 µl Rm – 0.5 µl	3 µl
Master Mix	Premixed	20 µl
	Final volume	25 µl

Results

Patient groups: In the current study, all the suspected MPN patients show hematocrit elevation for PV,whilethe hematocrit level for other MPNs were decreased, and most of the patients have had splenomegaly phenotype (questionnaire data). Samples were collected from 100 individuals, who suspected as MPN patients. These patients were randomly picked, whose ages ranged between 19-81 years old. The gender distributionwas50% male and 50% female (Figure 1). Moreover, the registry of these 100 patients were indicated for those lived in urban or rural of Sulaimani province (Figure 1).

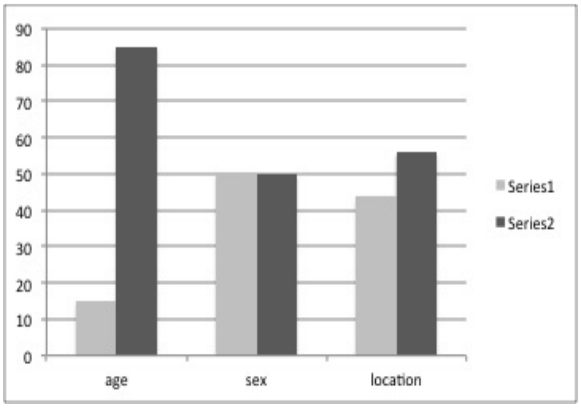


Figure 1: Distribution of patients according to age, gender and locality for all 100 patients. The age group, (series1) indicates that 15% of patients was below 40 years old and (series2) shows 85% to be above 40 years old. For gender group, Female (series1) and Male (series2) are equally distributed (50%). For locality distribution, (series1) indicates 44% of the patients who live in urban area and (series2) indicate that 56% of patients live in rural areain Sulaimani Governorate.

Genomic DNA: Genomic DNA for a number of samples were visualized in Figure 2 after the extraction from blood samples and the rest of the samples had similar or better quality of genomic DNA. The purity of human genomic DNA of all the samples indicates the good quality needed for subsequent *JAK2* amplification with multiplex PCR process (not shown).

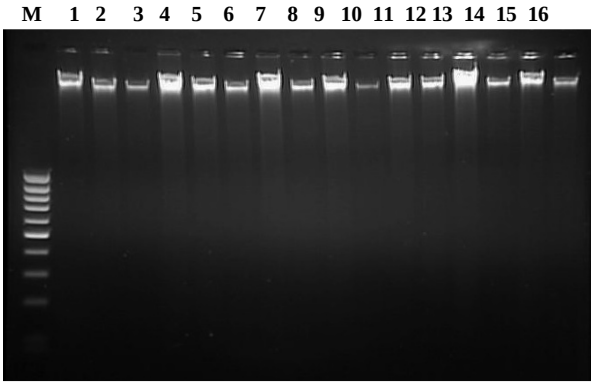
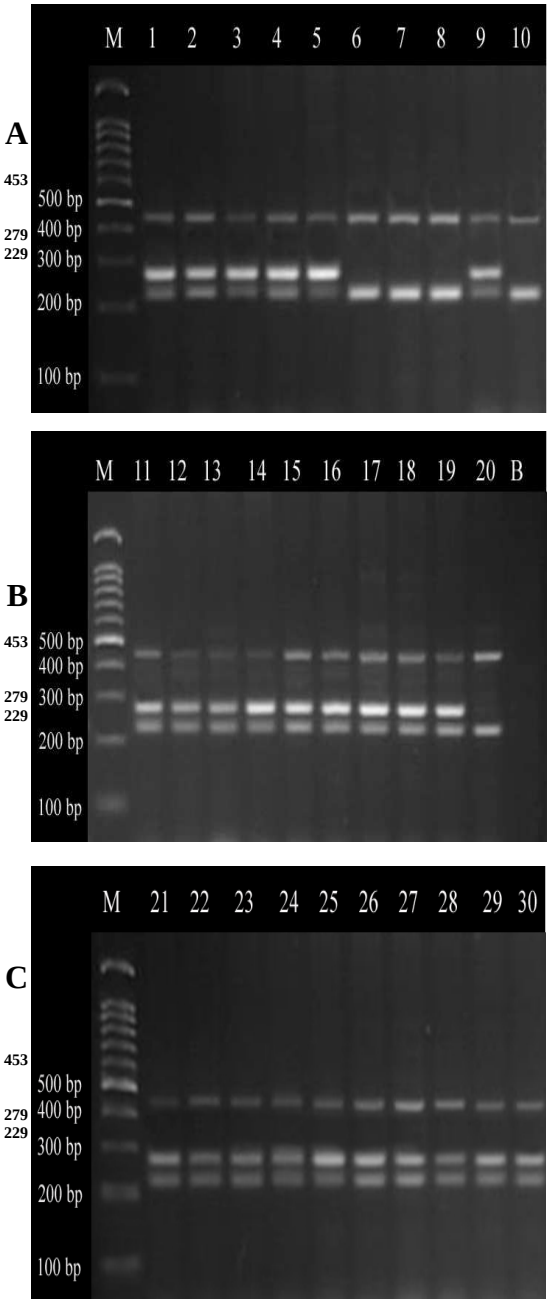


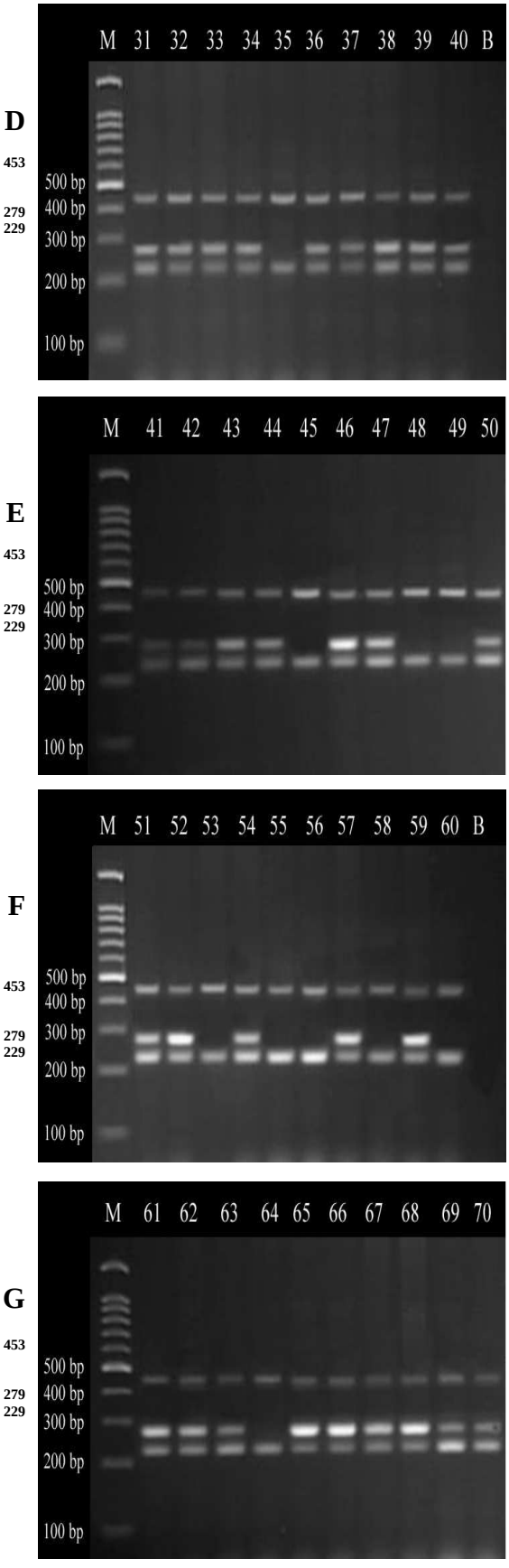
Figure 2: Extraction of genomic DNA from patient whole blood visualizedon agarose gel. M is an indication DNA marker (ladder), lanes 1-16 reveals genomic DNA samples of 16 patients (selected randomly) that was suspected with

MPNs. The results for other patients were equivalent to these data.

Genetic analysis; Mutiplex PCR was the main practical tool for *JAK2* amplification and *JAK2* c.1851G>T (p.V617F) mutation detection in this study.Multiplex PCR technique was conducted for *JAK2* gene in exon 14, chromosome 9 by using two sets oligonucleotide primers. Three fragments were amplified for each samples of all 100 patients and the results shown in Figure 3, were 65% was positive (+ve) for *JAK2* c.1851G>T (p.V617F) mutation with 46.15% male and 53.85% female; also 35% were negative for *JAK2* c.1851G>T (p.V617F) mutation with 57.14% male and 42.86% female.

Three fragments of DNA in exon 14 was amplified that is the *JAK2* c.1851G>T (p.V617F) mutation responsible for and lead to the production of JAK2 protein with subsequent MPNs; (first band as an internal positive control fragment with 453 bp size for both chromosomal alleles of the gene. For the positive mutation site, a PCR product of 279 bp size will be generated for the mutation allele. The third band is a 229 bp size product for normal (wild-type) allele alone, whether the normal *JAK2* is transcribed on other allele or indicate the homozygous of the wild-type on both alleles, indicating the absence of the c.1851G>T mutation.




D
M 31 32 33 34 35 36 37 38 39 40 B
453
279
229
500 bp
400 bp
300 bp
200 bp
100 bp
E
M 41 42 43 44 45 46 47 48 49 50
453
279
229
500 bp
400 bp
300 bp
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100 bp
F
M 51 52 53 54 55 56 57 58 59 60 B
453
279
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500 bp
400 bp
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100 bp
G
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453
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229
500 bp
400 bp
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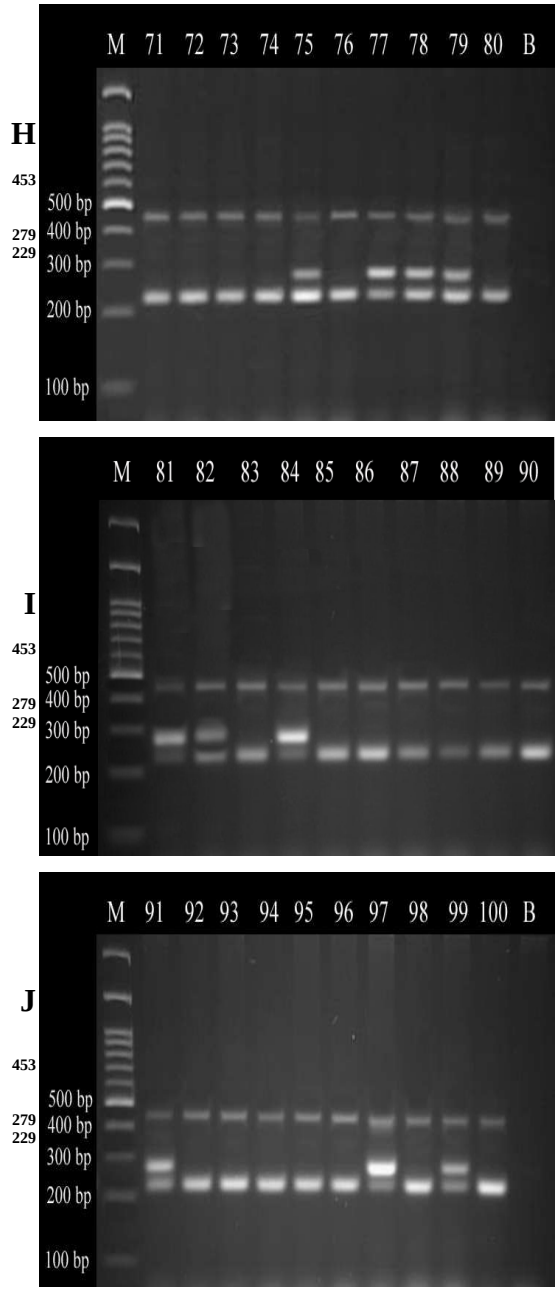

Figure 3: ARMS analysis for *JAK2*c.1851G>T (p.V617F)-mutation, for the whole group of patients (A-J). The multiplex PCR were run with 2 sets of primers as described in materials and methods. M; indicated DNA marker (100 bp ladder). Lanes arranged in numbers (1-100), corresponding to patient ID number. Reagent blank is indicated as a negative PCR control (B). 2% agarose gels were prepared on mini-agarose gel unit (APELEX, France) and the electrophoresis was run for 85 min on 90V. The bands were visualized using 2-drops EthBr per 100 ml TBE buffer. The data were collected on gel documentation system(Major science, Taiwan)

Discussions

In this study, a somatic mutation of *JAK2* c.1851G>T (p.V617F) was screened for a point mutation G>T mutation located in exon 14, which leads to single nucleotide replacement (missense mutation) at position 1851. At this position, the amino acid valine is substituted to phenylalanine at codon 617 in the JH2 domain (Kilpivaara and Levine, 2008).

Although the occurrence of *JAK2*c.1851G>T (p.V617F) mutation greatly contributed towardprecise diagnosis of MPNs, it remains unclear why this single genetic change represents at least three clinical phenotypes (i.e., PV, ET, and PMF) (Kim *et al*., 2013). It is always difficult to correlated phenotype with genotype, since the genetic variations of other genes or the same gene family leads to compensatory protein

function in cells.

However, several hypotheses have being proposed regarding how a single mutation can be contributed to three clinical phenotypes, but they are still under investigation in order to identify: (1) differences in the targeted hematopoietic stem cells, (2) define host modifier polymorphisms, (3) components of *JAK2* c.1851G>T (p.V617F) signaling, presence of other or additional somatic mutations, or the presence of a upstream signaling event that may vary according to the MPN phenotype (James, 2008). However, these hypotheses have to be validated.

Other advanced techniques, such as pyrosequencing, qPCR, denaturing high performance liquid chromatography (dHPLC) and capillary electrophoresis have been successfully employed for an exact detection of the *JAK2* mutant allele (James, 2008). These techniques are accurate and sophisticated for *JAK2* c.1851G>T (p.V617F) mutation screening, but they require expensive equipment and reagents, which might not be easily available or affordable in all laboratories. For these reasons, ordinary multiplex PCR techniques using a set of four primers, developed for ARMS assay, are less expensive; they only require conventional PCR instruments to provide sufficient sensitivity for routinely diagnostic purposes of *JAK2* c.1851G>T (p.V617F) mutation detection. This was our strategy to approach missense mutation analysis in this study.

In other studies, direct sequencing technique for *JAK2* mutation analysis has been used. However, the DNA sequencing technique is labor extensive, tedious and more expensive, when compared to allele specific oligonucleotide (ASO) PCR or amplification refractory mutation system (ARMS) PCR techniques (Koksal *etal*., 2007;Lieu *et al*., 2008). In comparison, the multiplex ASO PCR assay that was used in this study is easy, cheap and reliable. The multiplex primer for conventional PCR discriminate the existence of both the wild type and mutant alleles in the same assay and also evaluate the full PCR product bands together with an internal control amplicon.So, the ASO-PCR assay differentiates precisely between homozygous and heterozygous allele carrying the missense c.1851G>T mutation with the wild-type alleles. Therefore, this procedure is definitely a reliable screening test/diagnosis for mutant homozygocity or heterezyosity mutation in patient groups suspected for MPNs and also healthy groups.

In addition, some studies use restriction fragment length polymorphism (RFLP) method as described by *Baxteret al,* (2005)relying on restriction enzyme *BsaXI* of the PCR products. Unfortunately, this technique has some disadvantages when compared to the methods described in the present study, due to incomplete enzymatic cleavages. The partial digestion of the wild-type PCR product will complicate the analyses analysis, because *BsaXI* is a type IIB restriction enzyme having the capacity to cleave and methylate the DNA sequences as well. A study by *Poodt et al*,(2006) has revealed that the recognition site can be either cut wild-type or methylate sequences. This is the key reason why the *BsaXI* digestions never reached homogenous completion.

Sattler et al, (2006) uses denaturing high performance liquid chromatography (dHPLC) assay for mutation diagnosis. However, the assay sensitivityfrom patientblood cells was not assessed, because samples must be taken from the bone marrow and also additional procedure has to be applied, such as reverse transcription step and the post PCR step. Indeed, this assayis more complex than the method used in the present study. This complexity is due to the isolation of RNA from hematopoietic stem cells (HSCs) in the bone marrow and the synthesis of cDNA library by means of reverse transcription. So the success of the PCR step rely on good quality cDNA library instead of genomic DNA. After this step, the PCR products from patient samples are mixed with control PCR products containing the wild-type *JAK2*, prepared from healthy individuals. For these reasons that making the technique more difficult and sometime false results observed.

In the present study, positive samples for the *JAK2* c.1851G>T (p.V617F) mutationare heterozygous, i.e. contain a mutant and a normal allele. We can discriminate the presence of a single allele *JAK2* c.1851G>T (p.V617F)mutation in a population of MPN suspected patients. Thus, it is reliable and validated for genetic screening.

Results from a recent Japanese study by Ismael *et al,* (2012) showed that childhood incidence of *JAK2* c.1851G>T (p.V617F) mutation in ET and PV is lower than adulthood forms. In our study, we did not find

any case of pediatric patients suspected MPNs, this indirectly indicates of lower incident of this missense mutation of *JAK2*in children.A study conducted by Ha *et al, (*2012)could observe only a few associations of the (c.1851G>T) allele with MPN phenotype. Results of the study indicate that the mutational incidence or alleleburden of (c.1851G>T) affects the severity of MPN-associated clinical and hematological phenotypes. This typical mutation is more likely to produce a more severe phenotype (e.g., elevated myelopoiesis or high frequency of other complications).

Therefore, our ARMS (ASO-multiplex PCR) technique has proven to be cost effective for determining the presence of *JAK2* c.1851G>T (p.V617F) mutation. Hence, the molecular diagnosis of MPNs is rather simple and inexpensive. The acquired c.1851G>T mutation is a good molecular marker, unambiguously establishes the presence of clonal disorders. We believe that *JAK2* mutation screening is essential and will rapidly become a frontline test for individuals with a suspected diagnosis of an MPN.

Conclusions

In our laboratory settings, the PCR protocol for identifying the *JAK2* c.1851G>T (p.V617F) mutation using ARMS (ASO-multiplex PCR) strategy was successful and reliable as diagnostic procedure. Implementing the molecular analysis for this unique mutation allele for the first time using specific multiplex primers. The prevalence of this mutation implies that *JAK2* c.1851G>T (p.V617F) mutation is an important marker for diagnosis, specifically for patients with MPNs. We observe that the *JAK2* c.1851G>T (p.V617F) mutation is linked to leukocytosis, high red cell mass and splenomegaly. The phenotypic non-malignant myeloproliferative neoplasms could be matched with the genotype in the presence of *JAK2* c.1851G>T (p.V617F) mutation. Identification of this mutation is extremely important for proper management.Additional molecular analysis of the suspected MPN patients is required, when the*JAK2* c.1851G>T (p.V617F) mutation in exon 14 is abscent. One of the recent focuses is to screen exon 12 of *JAK2* gene, an additional mutations have been observed in recent years. Thus screening for other mutations, other than *JAK2* c.1851G>T (p.V617F) mutation, is important for precise diagnosis in suspected MPN patients,when*JAK2* c.1851G>T (p.V617F) mutation in exon 14 is absent (negative).

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