Original Article

Detection of JAK2V617F tyrosine kinase mutation and estimation of serum erythropoietin in blood donors who have high hematocrit

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Summary:

Background: JAK2V617F mutation is the most prevalent molecular abnormality in myeloproliferative neoplasms (MPNs) and has become a valuable marker for diagnosis of MPNs. Almost all patients with polycythemia vera (PV) have this acquired mutation. However, it has also been found in many other hematological diseases, and some studies even detected the presence of JAK2V617F in normal blood samples.

Objectives: To discuss the actual need to defer blood donors with high hematocrit.

Patients and methods: This prospective case control study was started on 16th of December 2012 and completed on 8th of July 2013, and enrolled 94 male blood donors who attended the National Blood Transfusion Center (NBTC) in Baghdad, Iraq. Their age range was between 21 and 62 years, and their hematocrit was ≥ 0.48 l/l. A control group of 20 patients known to have JAK2V617F positive PV were also included. The following investigations were done for both the study and the control groups: complete blood count, serum erythropoietin, and real time PCR for JAK2V617F detection.

Results: The present study found that 21.3% (20/ 94) of healthy blood donors were positive for JAK2V617F mutation. The mutant ratio was higher in the PV control than positive JAK2V617F donors (p=0.001). Out of 20 donors who have positive JAK2V617F mutation, 90% were smokers, and 22.8% (18/79) of all smokers were positive for JAK2V617F, while 13.3% (2/15) of non-smokers showed positivity for the mutation, the difference between various types of smoking and the level of JAK2V617F mutation among study groups was statistically insignificant with a p-value of 0.356. There is a frequent association of pruritis with JAK2 positivity as the percentage of pruritis in JAK2V617F positive blood donors was 40%. There was significant statistical difference between donors with positive JAK2V617F mutation and the PV control group for the hematocrit, total WBC count, absolute neutrophil count, and platelet count with p-values of (0.002), (0.001), (0.001), and (0.001) respectively.

Conclusion: The frequency of JAK2V617F mutation was much higher than that anticipated for blood donors. The blood donated from individuals with upper normal hematocrit does not necessarily denote to complete safety of blood as being devoid of JAK2V617F mutation.

Keywords: Blood donors, polycythemia vera, real time PCR, JAK2V617F.

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Introduction:

It is not uncommon to observe in blood donors a hematocrit (Hct) or hemoglobin (Hb) value above or close to the upper limit of normal, the clinical evaluation and diagnosis may be complicated by regular blood donations that can mask an underlying disease. Such values have been investigated to exclude or confirm the presence of secondary causes of polycythemia as well as a primary MPN such as PV.(1)

True polycythemia is a condition that results in an increased level of circulating red blood cells (RBCs) in the blood stream, which usually manifests itself as raised Hb concentration and/or Hct, while relative polycythemia is due to a reduction in plasma volume without an increase in total red cell mass (RCM). True polycythemia can be further subdivided into two categories; primary and secondary. Patients who cannot be assigned to either primary or secondary polycythemia are grouped together under the category of idiopathic erythrocytosis.(2,3)

Most patients with primary polycythemia have PV, an acquired MPN.(4,5) Secondary polycythemia, is due to aberrant regulation of erythropoiesis promoting substances, mainly erythropoietin (EPO), that act on erythroid progenitors.(6) Secondary polycythemia can be driven by various causes of hypoxia resulting in physiologically increased EPO production. (7).

EPO is hematopoietic growth factor that is essential for the effective differentiation and maturation of erythroid

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progenitors. Its production is increased in anemia or in hypoxia. Estimation of serum EPO level could be potentially helpful in distinguishing between the different types of polycythemia. (2,8)

PV is characterized by excessive proliferation of erythroid, myeloid and megakaryocytic elements in the marrow, increased cell count in peripheral blood and increased erythroid mass. The cause of PV is not fully understood, in 2005, several groups identified that almost all patients with PV have an acquired mutation of the Janus associated kinase 2 (JAK2) gene on chromosome 9 and is found in the majority of patients with MPNs and has become a valuable marker for diagnosis of MPNs. However, it has also been found in many other hematological diseases, and some studies even detected the presence of JAK2V617F in normal blood samples.(3,9)

Because the JAK2V617F mutation may be present only in a small proportion of cells, sensitive detection methods are required.(10) There are various techniques used to detect the mutation, one of them is polymerase chain reaction (PCR). Using PCR; specific sequences within a DNA can be amplified many thousand to a million fold. Quantification of the amplified product is obtained using fluorescent probes or fluorescent DNA binding dyes and real-time PCR instruments that combines thermal cycling with scanning capability. (11,12)

Patients and methods:

This prospective case control study was started on 16th of December 2012 and completed on 8th of July 2013, and enrolled 94 male blood donors who attended the NBTC. Their age ranged between 21 and 62 years, and their Hct was ≥ 0.48 l/l, and subsequently, those with Hct higher than 0.55 l/l were deferred from donation but included in the study. This study also included a control group of 20 patients known to have JAK2V617F positive PV, who were diagnosed according to the British Committee for Standards in Haematology (BCSH) criteria. Blood of 15 healthy Iraqi individuals were tested to set the upper cutoff normal mutant ratio of JAK2V617F for this study (which was shown to be 1.3%). History of smoking, pruritis, bleeding episodes, was taken from the blood donors and none gave any history of chronic lung disease or thrombotic tendencies. About 8 ml of venous blood was withdrawn from each donor and control, and divided in 3 tubes; a plain tube for serum EPO level estimation in addition to two K2EDTA-containing tubes, one for complete blood counts and the other for molecular analysis.

Blood donors were also divided into two groups according to the presence (20) or absence (74) of JAK2V617F mutation.

Serum samples were tested for EPO by enzyme linked immunosorbent assay (ELISA) using Quantikine IVD Human EPO Elisa kit /R&D System, Inc USA, which depends on a normal range for serum EPO of (2.5–200 mIU/ml).

DNA was extracted by a phenol/chloroform method.(13) A quantitative real time PCR (qPCR) was performed using AccuPower®JAK2V617F Quantitative PCR kit and an Exicycler 96 device from Bioneer with a sensitivity level of 0.01%. The principle of the method used by the kit for the detection of JAK2V617F mutation is Allele specific oligonucleotide (ASO), the number of the primers included in the reaction were 2 for each tube (one tube detects a wild type and the other tube detects a mutant type of JAK2), real-time fluorescence signal was detected by the use of fluorescent sequence-specific probes (TaqMan probes). Reaction conditions, based on manufacturer's instructions were; a pre-denaturation at 95°C for 10 minutes followed by 45 cycles of denaturation at 95°C for 20 seconds, annealing and extension at 55°C for 30 seconds. Quantitation of the mutant and wild alleles was done using Exigenotypetm software, which automatically calculates the concentration of the wild and the mutant alleles and the mutant to wild allele (M:W) ratio.

Statistical analysis: was done using SPSS version 21, t-test was used to compare continuous data. Association between categorical data was done using Chi square test, and one way ANOVA test was used to compare continuous data among the three groups of variables. P value <0.05 was considered significant.

Results:

The present study found that 21.3% (20/94) of blood donors were positive for JAK2V617F mutation. The study revealed higher counted mutant ratio of JAK2V617F in PV patients than blood donors with positive JAK2V617F mutation with a mean (%) value of 58 ± 26.9 (range >1.3 - 100) versus 11.10 ± 12.6 (range >1.3 – 42.6), respectively, and there is a highly significant statistical difference between both groups (p-value = 0.001), figure (1).

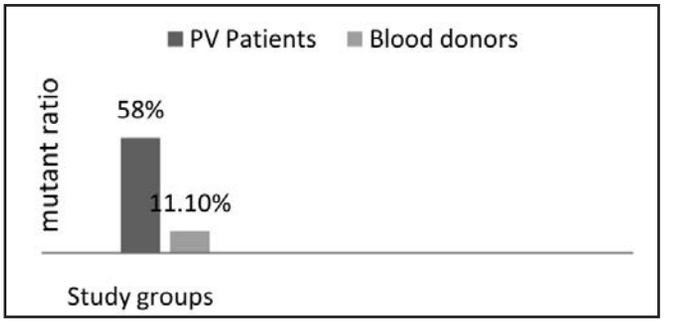

Figure 1. The mean JAK2V617F mutant ratio of 20 PV patients and 20 blood donors

About 84% (79/94) of blood donors who came to the NBTC were smoking cigarettes, hookah or both and one of the reasons for their blood donation was high normal Hct. In this study; out of 20 donors who have positive JAK2V617F mutation, 90% were smokers, and about 22.8% (18/79) of all smokers were positive for JAK2V617F, while around 13.3% (2/15) of non smokers showed positivity for the mutation. However, the difference between various types of smoking and the level of JAK2V617Fmutation among study groups was statistically insignificant with a p-value of 0.356 (Table 1).

Table 1. Differences between the types of smoking, and non smokers in accordance with the results of JAK2V617Fmutation among the study population.

JAK2V617F results	Blood donors according to smoking Type						Non smokers	%	p-value(2)
	C(1)	%	H(1)	%	C&H(1)	%			
Positive n=20 (21.3%)	13	65	2	10	3	15	2	10	0.356
Negative n=74 (78.7%)	44	59.5	11	14.9	6	8.1	13	17.5	
Total	57		13		9		15		

(1) C, cigarette; H, hookah
(2) Statistic done by using ANOVA test

The smoking index can be calculated in only 66 blood donors (57 persons smoke cigarettes and 9 persons smoke both cigarettes and hookah). Two groups of smokers were categorized according to their smoking index. The frequency of JAK2V617F positivity is almost doubled in smoking index group ≥ 10 pack year constituting 28.0% than < 10 pack year group (12.5%), however, the association between the smoking indices for these groups and the JAK2V617F status was statistically insignificant with a p-value of 0.319 (Table 2).

Table 2. Groups of smokers according to their smoking index with JAK2V617F mutant status.

Smoking index (pack year)	Negative JAK2V617F	Positive JAK2V617F	Total	(%) of +ve JAK2V617F	p-value*
< 10	14	2	16	12.5 %	
≥ 10	36	14	50	28 %	0.319
Total	50	16	66		

*Statistic was done by using Chi square test

There was significant association of pruritis with JAK2 positivity as the percentage of pruritis in JAK2V617F positive blood donors was 40% which almost resemble that of PV control (45%), and there was no significant statistical difference between these two groups with p-value of 0.752 (Table 3).

Table 3. The frequency of pruritis in positive JAK2V617F donors and PV control group.

+ve JAK2 groups	Pruritis		Total	p-value*
	No Pruritis	Pruritis (%)		
Donors	12	8 (40%)	20	
PV Controls	11	9 (45%)	20	0.752
Total	21	19	40	

*Statistics was done by using Chi square test.

None of the blood donors had a history of thrombosis, while comparing bleeding episodes between positive JAK2V617F donors, in which 2 donors had history of recurrent epistaxis, and the control group of PV patients, in which 3 patients gave bleeding history (one with epistaxis and the other 2 with GIT bleeding), the difference was found statistically insignificant (p-value = 0.63).

There was insignificant difference for the subnormal level of EPO between positive and negative JAK2V617Fmutation blood donors groups (p-value = 0.151), and between the control group of PV patients and the positive JAK2V617F donors (p-value = 0.82).

Hematological parameters; Hct, RBC count, total WBC count, absolute neutrophil count (ANC) and Platelet count: the differences between JAK2V617F positive and negative mutation in blood donors were statistically insignificant with p-values of 0.879, 0.113, 0.981, 0.875, and 0.126 respectively. The same hematological parameters were used to compare between the positive donors with JAK2V617F mutation and the control group of PV patients; the differences were statistically significant for Hct, WBC count, ANC, and platelet count with p-values of 0.002, 0.001, 0.001, and 0.001 respectively, while for RBC count the difference was statistically insignificant with p-values of 0.589 (Table 4).

Table 4. The hematological parameters in blood donors with positive JAK2V617Fmutation and the PV control groups.

Parameters	+ve JAK2 Groups	Total No.	Mean ± SD	p-value*
Hct (%)	Donors	20	52.5 ± 3.9	0.002
	Control	20	56 ± 2.5	
WBC (×109 /L)	Donors	20	9.7 ± 2.7	0.001
	Control	20	19.3 ± 8.7	
ANC (×109 /L)	Donors	20	6 ± 2.1	0.001
	Control	20	15.7 ± 11.1	
Platelets (×109 /L)	Donors	20	331 ± 124.6	0.001
	Control	20	556 ± 207.9	
RBC (×1012 /L)	Donors	20	5.9 ± 0.4	0.589
	Control	20	6 ± 0.3	

*Statistics was done by using t-test

Discussion:

JAK2 mutation: JAK2V617F is often present in patients with chronic Philadelphia-negative classical MPNs, characterized by an elevated Hct and/or WBC. The mutation has also been reported in the assumed healthy individuals who are confirmed to be without a known hematologic disease,(14) the present study also found that 21.3% (20/94) of healthy blood donors were positive for JAK2V617F mutation. The difference between mean mutant ratios between positive JAK2V617F donors and the PV control was statistically significant with p-value of = 0.001 (Figure 1).

Studying the presence of JAK2V617F in healthy subjects has been the focus of some studies, the largest study was that of Xu X et al.(14) which applied nested PCR and was able to detect JAK2 in 0.94%, and JAK2 was detectable in them at levels far exceeding the 1% cutoff level. Another study, by applying ASO qPCR, Rapado I et al.(15) was able to detect the mutation in 2% of normal healthy individuals and in 2 of them the level was (3.2% and 4.2%).

These results are far less than what is reported in present study for the following reasons; First: this study included 14 blood donors who had Hct $\geq$ 0.55 and were subsequently deferred and hence they are not considered as healthy donors (5/14 were positive for the mutation). Second: 12/94 blood donors (12.8%) had Hct $\geq$ 0.52 and were positive for JAK2V617F mutation, and these donors should be classified as PV patients according to BCSH criteria.

Nevertheless, even if we exclude the 12.8% of those who should be diagnosed as PV patients there will remain 8.5% of supposed healthy blood donors with positive JAK2V617F mutation, a percentage which is still much higher than reported by other studies. This raise the actual need to establish a national normal reference range for Hb and Hct which is supposed to be less than western reference values, and that our patients with positive JAK2V617F mutation may be regarded as PV patients at Hct levels less than 0.52 l/l defined by the BCSH criteria.

There are many hypotheses for this high percentage among the assumed healthy blood donors. One hypothesis is that; discovering the mutation in healthy subjects raised the possibility that the mutation is harbored before the occurrence of the chronic MPNs. This should be confirmed by an adequate follow-up of healthy subjects who carry the mutation.(16) Another hypothesis; the JAK2V617F in healthy subjects is due to the first progenitor to harbor JAK2V617F might be more differentiated in healthy subjects than in patients with a chronic MPN. At this stage, the self-renewal potential and the capacity to differentiate the mutated cell are decreased and the clone it generates might be prone to die. In this hypothesis, the mutated clone does not generate a clinical phenotype and it may disappear at serial evaluation. Conversely, in PV and in myelofibrosis, the JAK2V617F mutation is harbored in a lympho-myeloid progenitor cell.(17)

Smoking: there were no significant differences between JAK2V617F positive or negative mutation donors (Table 1) and between the two groups of JAK2V617F positive mutation donors regarding their pack year levels (Table 2) with p-values of 0.356 and 0.319 respectively. However, interestingly, there is 1.7 fold increases in JAK2V617F mutation in smokers as about 22.8% (18/79) of smokers were positive for JAK2V617F, while around 13.3% (2/15) of non-smokers showed positivity for the mutation (Table 1). As well; there is 2.24 fold increase in incidence of JAK2V617F positivity with smoking index group $\geq$ 10 pack year constituting 28% than < 10 pack year group, 12.5% (Table 2). This may signify the risk of possible contribution of smoking and its amount to the emergence of JAK2V617F mutation.

Ido Weinberg et al. study(18) suggests a similar correlation between the smoking habits and the frequency and prevalence of JAK2V617Fmutation, possibly related to the poor DNA repair as a result of the increased demand for the erythrocyte production.

Pruritus occurs in approximately 40% of PV patients. It tends to be more severe when the disease is active and becomes milder or disappears when control is achieved by myelo-suppression.(19)

There was no significant statistical difference for the presence of pruritis when compared between positive JAK2V617F donors and the control group of PV patients (p= 0.752, table 3). This may reflect the effect of JAK2V617F mutation and high Hct in both groups on pruritis. In our study percentage of pruritis in PV control was 45% which is close to the percentage in JAK2V617Fpositive blood donors (40%), both are certainly much different than in normal healthy populations with a negative mutation and lower Hb levels. JAK2V617F mutation may be the early parameter that changed during the course of the suggested MPN including PV.(21)

The difference of bleeding episodes between positive JAK2V617Fmutation donors (2 donors presented with recurrent epistaxis) and the control group of PV patients (one with epistaxis and two presented with upper GIT bleeding) was insignificant (p= 0.63). This goes with Andrea Patriarca et al. study that couldn't verify or decline the association of JAK2V617F positivity and its protective role on the bleeding risk and suggests the need to large studies to determine this correlation.(22)

Analysis of serum EPO level showed that there was no significant difference found between the two groups of blood donors in relation to JAK2V617F mutation (p= 0.151), also there was no significant difference between positive donors for JAK2V617F and the positive control group of PV patients (p=0.821). These results in contrast with Pascal Mossuz study who reported that the mean EPO level was significantly lower

in patients with PV and this difference may be attributed to the timing of measurement of serum EPO level of PV controls which was done after diagnosis and treatment of PV patients (phlebotomy and/or chemotherapy) and most of their EPO level were probably returned to normal. Decreasing RCM influences serum EPO level and therefore absence of previous treatment including phlebotomy is of major importance if serum EPO level is to be used as a diagnostic criterion of PV at initial presentation, however a normal serum EPO concentration does not exclude the possibility of PV and even slightly raised EPO values have been observed in PV.(23-25) Lowered EPO level was encountered in 5.4% of JAK2V617F negative blood donors, this may in fact point to relatively increased erythrocytosis in smokers with resultant down-regulation of EPO secretion.(18) As well, EPO is only decreased in 10% of JAK2V617F positive blood donors. One would expect higher level of EPO in smokers, as a contributing factor of their secondary polycythemia. This controversial finding may not satisfy the inclusion of subnormal EPO level as a WHO minor criterion for diagnosing PV.

For the hematological parameters (Hct, RBC count, total WBC count, ANC and Platelet count), the differences between JAK2V617F positive and negative mutation in blood donors were statistically insignificant with p-values 0.879, 0.113, 0.981, 0.875, 0.126 respectively. This is consistent with the results of other studies,(14,18) which indicates that Hct alone cannot be regarded as an isolated marker to follow up donors of blood for JAK2 mutation. Although around 30% of donors in JAK2V617Fpositive and negative groups showed an increase in WBC count and 20% showed an increase in ANC, this may reflect little impact of JAK2V617F positivity on leukocytosis, probably because of the low mutant burden ratio (11.1%), on early laboratory changes. Therefore the leukocytosis may be more likely attributable to the effect of smoking on hematological parameters (as 18 out of 20 JAK2V617F positive donors were smokers, table 1), both active and passive cigarette smokers have a significant effect on many hematological normal reference values including WBC and ANC. Some effects may be transient and their severity varies between individuals as well as by the number of cigarettes smoked. Smoking 10 cigarettes per day result in slight leukocytosis. The leucocyte count increases, largely, as a result of an increase in the neutrophils and neutrophil function may be affected. Leukocytosis presents in a wide variety of diseases, it cannot be dependable as a single parameter to denote to JAK2 mutation, as there may be an intercurrent illness causing leukocytosis during the donation period.(26)

The same hematological parameters were used to compare between donors with positive JAK2V617F mutation and the control PV group; the differences were statistically significant for the Hct, WBC count, ANC, and platelet count with p-values of 0.002, 0.001, 0.001, and 0.001 respectively. For RBC count, the difference was statistically insignificant (p-value 0.589, table 7). These findings are similar to the results reported by previous studies.(1,14,18,27)

Conclusion:

The frequency of JAK2V617Fmutation was much higher than that anticipated for individuals who are attending the NBTC for blood donation. Therefore; the blood donated from individuals with upper normal Hct does not necessarily being devoid of JAK2V617Fmutation and completely safe for recipients of blood transfusion. This raised the importance of routine testing and detecting JAK2V617Fmutation in completely healthy donors. The smoking risk to develop JAK2V617Fmutation is 1.7 fold than non-smokers, and heavy smokers with smoking index of $\geq$ 10 pack year shows 2.24 fold increase risk to develop JAK2 mutation.

Author contribution:

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