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Implications of Molecular Estimation of Allelic Burden of *JAK2 V617F* Mutant Allele in Essential Thrombocythemia and Polycythemia Vera Diseases

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Authors' contributions

This work was carried out in collaboration among all authors. Author PNM conceptualized designed and coordinated the study. Author BAS performed the optimization and validation, analysis of the samples, wrote the protocols. Authors BAS, KHG and HP acquired the data. Authors PNM, AA and BAS did the analysis and interpretation of data. Author KHG did the literature searches. Authors BAS and PNM prepared the manuscript. Author PNM revised the manuscript. Authors KHG, SV, AA and HP managed the statistical analyses of the study. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: Development of a JAK2 Allelic burden estimation kit using Taqman probes and determine trend in shifting of *JAK2 V617F* allelic burden in and its use in analysis of patients with essential thrombocythemia (ET) and polycythemia vera (PV) to predict thrombotic complications.

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Methodology: Through a retrospect study, a total of 412 ET and PV patient, divided into 3 groups (0-2, 2-5 and 5-10 years) based on time of detection of the disease, were tested in retrospect for presence of the *JAK2 V617F* mutant allele burden using an in-house developed Taqman probe-based kit, trend in shifting of the mutant allele burden was studied and segregated into ET (n=167) and PV (n=126) group based on their disease profile. It was then categorized into 3 time periods (0-2, 2-5 and 5-10 years) based on time of detection of the disease.
Results: Around 293 (71%) were positive for *JAK2 V617F* while 59 (14.2%) and 8 (1.9%) positive for CALR exon 9 frame shift & MPL mutations (W515L/W515K) respectively. The 1-25% allelic burden group size gradually fell in ET population over time and this trend continued in the PV population also. In the former the fall was 7% & 11% for 2-5 & 5-10 years category while in the later, it was 1% and 15% respectively for the same time period-category.
Conclusions: There is a distinct molecular continuum in the *JAK2 V617F* allelic burden in the ET & PV patients which followed a predictable trend and was associated with increasingly complicated vascular events.

Keywords: JAK2 V617F; thrombosis; essential thrombocythemia; polycythemia vera; allelic burden.

1. INTRODUCTION

Myeloproliferative neoplasms (MPNs) are clonal disorder of hematopoietic stem cells, characterized by enhanced proliferation of the myeloid lineage with effective maturation that ultimately lead to increased red blood cell mass [1]. The MPNs comprise of polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), which are also called breakpoint cluster region-Abelson fusion oncogene (BCR-ABL)-negative neoplasms [2]. These three diseases exhibit common feature such as growth factor-independent proliferation of hematopoietic stem cells, bone marrow hyper cellularity and an enhanced risk of thrombotic events and haemorrhages [3,4,5,6].

In the year 2005, mutation in JAK2 gene was identified within exon 12 that resulted in a 'g. 1849 G>T' alteration substituting a valine for a phenylalanine at amino acid position 617 of the protein [7]. This mutation is known to permanently activate the JAK/Signal Transducers & Activators of Transcription (STAT) signalling and allied downstream pathways leading to the disorder. The frequency of occurrence of *JAK2 V617F* mutation is 95% in PV and 55–60% in ET and PMF. In fact, The Janus kinase 2 (JAK2) V617F mutation is one of the most common molecular anomaly in the Ph- (Philadelphia chromosome negative) MPN variants in the population [8,9,10,11].

These three Ph-MPN subtypes present significant overlap in their clinical presentation, course of the disease and bone marrow histopathology [12]. The *JAK2 V617F* mutation assay is currently the molecular test of choice given that it is an important clonal marker for the diagnosis of Ph-MPN. But, importantly, its presence alone cannot discriminate the three different subtypes of Ph-MPN. Further still, it remains to be deciphered that how a single mutation can be found in all the three different disease subtypes of varying clinical presentation and prognosis.

The potential of V617F JAK2 mutation to revolutionize the way Ph-MPN are diagnosed and classified has led to development of some robust methods of its detection by allele-specific amplification of the mutant allele [13], restriction enzyme digestion based assay [8] and conventional nucleotide sequencing techniques [14]. All three techniques are implemented in different diagnostic laboratories as per their need and requirement [15].

In recent times, Real-time Quantitative PCR (RT-PCR) techniques has evolved to independently detect and quantify the wild and mutant allele of *JAK2 V617F* in a single reaction in Ph-MPN patients. Recent findings suggest that proportion of JAK2 mutant allele in Ph-MPN patients is closely associated with different disease phenotypes linked to subtypes of Ph-MPN. A higher *JAK2 V617F* allele burden is seen in PV than in ET [16,17,18], thus making quantitation of allelic burden important diagnostic activity. For example, Hussein and his co-workers demonstrated that a *JAK2 V617F* allele proportion of >50% supports diagnosis of prefibrotic PMF rather than ET [19].

In this study, we attempted to develop a *JAK2 V617F* allelic discriminatory kit using Taqman probes [20] and demonstrated its utility in

stratifying Ph- MPN patients which is otherwise not possible with older techniques of qualitative detection of this mutant allele [15].

2. MATERIALS AND METHODS

2.1 Resource Population

Genomic DNA extracted from 412 patients previously diagnosed for Ph-MPN and referred to SN GeneLab, India, were used in this study. Blood sample from healthy individual controls (n=12) obtained from SN GeneLab sample repository were used as control. Available patient's data were used to classify patients with *JAK2 V617F* mutation in retrospect into either PV or *ET* .

Broadly, platelet count of ≥450 x 109/L, bone marrow biopsy showing proliferation mainly of the megakaryocyte lineage with increased numbers of enlarged, mature megakaryocytes with hyperlobulated nuclei, no significant increase or left-shift in neutrophil granulopoiesis or erythropoiesis, very rarely minor (grade 1) increase in reticulin fibers and presence of JAK2, CALR (calreticulin) , or MPL (myeloproliferative leukemia) mutation were the major criteria while presence of a clonal marker or absence of evidence for reactive thrombocytosis were considered the minor criteria for diagnosis of *ET* Diagnosis required meeting all 4 major criteria or the first 3 major criteria along with the minor criterion.

For Polycythemia Vera, Hb >16. 5 g/dL in men, >16. 0 g/dL in women, or Hematocrit >49% in men, >48% in women, or increased red cell mass, bone marrow biopsy showing hypercellularity for age with trilineage growth (panmyelosis), including prominent erythroid, granulocytic, and megakaryocytic proliferation with pleomorphic, mature megakaryocytes (differences in size) >25% above mean normal predicted value and presence of *JAK2 V617F* or JAK2 exon 12 mutation were considered the major criteria while Subnormal serum erythropoietin level was considered here as the minor criteria. Diagnosis required meeting either all 3 major criteria or the first 2 major criteria along with the minor criterion.

2.2 DNA Extraction

DNA was extracted from the peripheral blood collected in EDTA vial within 24 hours from collection, using a Wobble Base Blood DNA Mini kit (Wobble Base Bioresearch, India) according to the manufacturer's protocol. The DNA concentration was determined using a Qubit 4 Fluorometer (Thermo Fisher Scientific, USA).

2.3 Quantitative Estimation of *JAK2 V617F* Using Taqman Probes

A polymerase chain reaction (PCR) assay employing TaqMan and minor groove binding probes were used for proportional quantitation of DNA copy number of *JAK2 V617F* wild-type and mutant alleles in clinical sample using a Rotor-Gene Q 2plex Platform (Qiagen, USA).

For generating quantitation standards for absolute estimation of V617F wild and mutant alleles, a 300 bp PCR product was generated from blood DNA of *JAK2 V617F* positive and negative patients respectively and cloned in E. coli (JM109) using a pGEM®-T Easy Vector (Promega Corporation, Madison, USA) as per manufacturer's instructions. Recombinant plasmid was extracted using a Wobble Base Plasmid DNA Extraction kit (Wobble Base Bioresearch, India) and the identity of the insert was thereafter confirmed by Sanger nucleotide sequencing.

For computing *JAK2 V617F* allelic burden the following formula was used:

Absolute copy number of the mutant allele / (Absolute copy number of the wild-type allele + Absolute copy number of the mutant allele) X 100

2.4 Genotyping for CALR and MPL Mutation

Patients harbouring negative (Wild-type) *JAK2 V617F* allele were genotyped for the JAK2 exon 12 mutations using the Sanger sequencing technique as previously described [21]. Patients with non-mutated JAK2 were interrogated for MPL exon 10 mutations (W515L and W515 K) using ARMS PCR as previously reported [22]. Exon 9 deletions within the CALR gene in non-mutated JAK2 and MPL patients were detected by Sanger sequencing as previously reported [23].

2.5 Nucleotide Sequencing of DNA Fragment Representing *JAK2 V617F* Wild-type and Mutated Alleles

Prior to molecular cloning, the authenticity of the PCR amplicon was confirmed by Sanger

sequencing method using the BigDye® Terminator kit, version 3.1 (Applied Biosystems Life Technologies, Foster City, CA, USA) as per manufacturer's instructions.

2.6 Primary and Secondary Calibration of the Recombinant Standards against Ipsogen JAK2 RGQ PCR Kit

For generating quantitation standards, purified recombinant plasmids were quantified using the following formula.

Number of copies = (amount of DNA in ng/µl X $6.022x10^{23}$) / (length X $1x10^{9}$ X 650)

Following this calculation, a set of 10^1, 10^2, 10^3, 10^4 and 10^5 copies/uL of quantitation standards were prepared and challenged against Ipsogen JAK2 RGQ PCR Kit for secondary calibration before being used in this study.

2.7 Generating the Standard Curve for Estimating the *JAK2 V617F* Mutant and Wild-type Allele

The Standard curve for estimating the absolute copy numbers of the mutant and the wild-type *JAK2 V617F* alleles was prepared by plotting the fluorescent signal from each dilution against cycle number. The quantity of standards (copies/µL) used were: 100000, 10000, 1000, 100 and 10 respectively for both the mutant & wild-type alleles.

2.8 Repeatability and Reproducibility of the Standard Curves

Repeatability of the standard curves were determined by setting up an RT-PCR for standard samples in triplicate from the same aliquot. Reproducibility of the standard curves were assessed by running separate aliquots of all five set of standards for the wild and mutant alleles in triplicate on three separate days by three different operators on a Roche, Rotorgene and ABI real time PCR machine respectively.

2.9 Chronological Distribution of Samples

All the *JAK2 V617F* positive samples were categorized into three different chronological time-periods based on the first time the patients were diagnosed for the disease. These time periods were 0 to 2 years, 2 to 5 years and 5 to 10 years respectively.

2.10 Statistical Analysis

All the laboratory parameters included in the statistical analyses were gathered at diagnosis. Differences in the distribution of continuous variables between categories were analyzed by either Mann–Whitney or Kruskal–Wallis tests. Patient groups with nominal variables were compared by the Chi-square or Fisher's exact test, when appropriate. P-values <0.05 were considered statistically significant. Statistical analyses were performed using the GraphPad Prism software, version 5. 5. 0 (GraphPad software, Inc., San Diego, CA, USA).

3. RESULTS

3.1 Construction of the *JAK2 V617F* Mutant and Wild-type Recombinant Plasmid

Two recombinant plasmids, namely pJAK-M and pJAK-W were constructed that harboured a 300 base pair *JAK2* gene fragment of the mutant (V617F) and the wild-type alleles respectively. Nucleotide sequencing of the insert of both the recombinant plasmids confirmed the identity of the clones (Data not shown).

3.2 Generating the Standard Curve for the Wild and Mutant Allele Estimation

Standard curves generated with the *JAK2 V617F* wild and mutant allele standards demonstrated repeatability and reproducibility with acceptable standard deviation. The repeatability results are summarized in Table 1 & Table 2 and illustrated in Fig. 1.

The reproducibility results are summarized in Tables 3 & 4 and illustrated in Figs. 2 & 3. For a standard curve to qualify for use in an assay, the slope and the r2 value were fixed at ~-3.3 and 0.99 for this study.

3.3 Genotyping for *JAK2 V617F*, CALR Exon 9 Frame Shift and MPL (W515L and W515 K) mutation

Out of the DNA from 412 patients, 293 (71%), 59 (14.2%) and 8 (1.9%) were found to be positive for the *JAK2 V617F* mutation, CALR exon 9 frame shift and MPL mutation respectively. The remaining 53 patients (12.8%) were triple negative.

Table 1. Repeatability of *JAK2 V617F* Wild-type allele

Standard (copies/µL)	Cycle threshold (Ct)*	Mean	Standard deviation
100000	14.02	14.06	0.08
	14.00		
	14.06		
10000	17.08	17.06	0.05
	16.99		
	17.11		
1000	20.17	20.04	0.089
	20.01		
	19.96		
100	22.6	22.56	0.071
	22.46		
	22.62		
10	26.13	26.03	0.108
	26.08		
	25.88		

Table 2. Repeatability of *JAK2 V617F* Mutant allele

Standard (copies/µL)	Cycle threshold (Ct)*	Mean	Standard deviation
100000	13.8	13.91	0.087
	14.01		
	13.94		
10000	16.53	16.38	0.108
	16.33		
	16.28		
1000	19.63	19.63	0.065
	19.55		
	19.71		
100	23.54	23.51	0.08
	23.59		
	23.4		
10	26.96	27.143	0.181
	27.39		
	27.08		

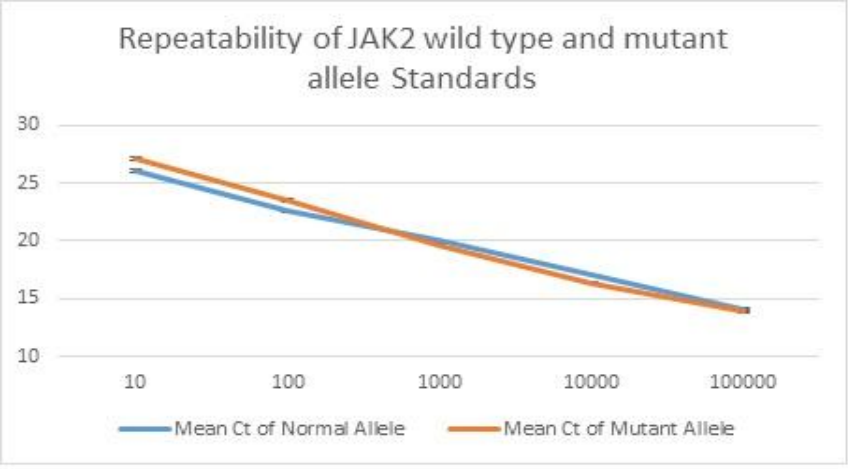

Fig. 1. Repeatability of JAK2 wild-type allele and mutant allele standards

Table 3. Reproducibility of *JAK2 V617F* Wild-type allele

Standard (copies/µL)	Cycle threshold (Ct)	Mean	SD	Cycle threshold (Ct)	MeanSD	Cycle threshold (Ct)	Mean	SD
	Rotorgene			ABI		Roche		
100000	14.02	14.06	0.08	16.08	16.000.065	15.12	15.06	0.196
	14.00			15.92		15.27		
	14.06			16.01		14.8		
10000	17.08	17.06	0.05	19.01	18.870.11	17.87	17.97	0.071
	16.99			18.74		18.03		
	17.11			18.88		18.01		
1000	20.17	20.04	0.089	22.76	22.770.159	20.72	20.92	0.143
	20.01			22.97		20.99		
	19.96			22.58		21.05		
100	22.6	22.56	0.071	24.31	24.470.138	23.37	23.51	0.101
	22.46			24.47		23.61		
	22.62			24.65		23.55		
10	26.13	26.03	0.108	28.2	27.940.182	26.98	27.14	0.153
	26.08			27.84		27.35		
	25.88			27.79		27.11		

SD: Standard Deviation

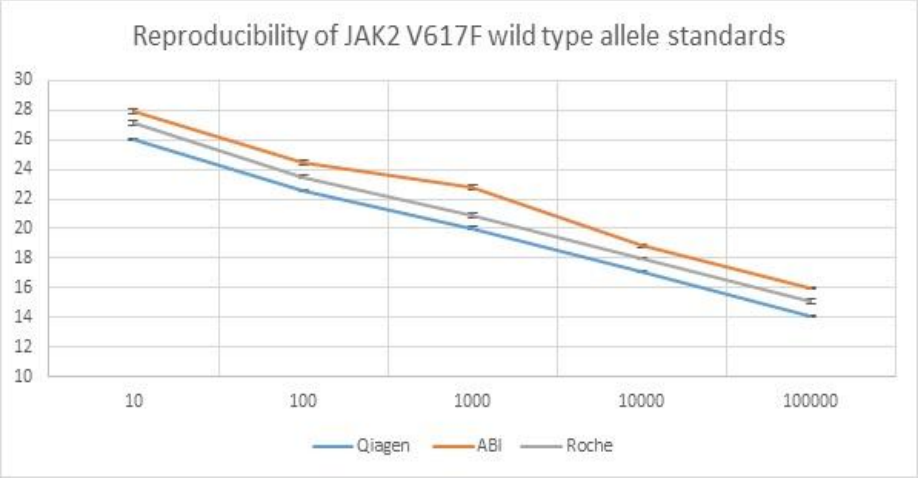

Fig. 2. Reproducibility of *JAK2 V617F* wild-type allele standards

3.4 Retrospect Chronological Classification of *JAK2 V617F* Positive Patients into PV and ET

Out of a total of 293 *JAK2 V617F* positive samples, 68, 46 and 53 belonged to patients who were diagnosed for ET at a time between 0-2, 2-5 and 5-10 years respectively, since the date of collection of samples. Similarly, 51, 47 and 28 patients were diagnosed for PV at a time between 0-2, 2-5 and 5-10 years respectively. The data is compiled in Table 5a and Table 5b. The spread of 3 levels of allelic burden (0-25%, 25-50% and >50%) is shown in Table 6 and illustrated in Fig. 4.

It was noted that the 1-25% *JAK2 V617F* allelic burden in the ET patient group gradually reduced with increasing time period since the date of detection and this trend of gradual reduction in the allelic burden value continued in the PV patient group also (Fig. 5a & Fig. 5b). But when seen together, there is a distinct and unequivocal continuum of this trend observed wherein the lowering percentage of the 1-25% allelic burden value in the 5-10 years block in ET patients smoothly transited into a still lower value of 21% in the 0-2 year block of the PV patients that further reduced to 20% and 5% in the 2-5 and 5-10 years block of the PV group respectively (Fig. 5a).

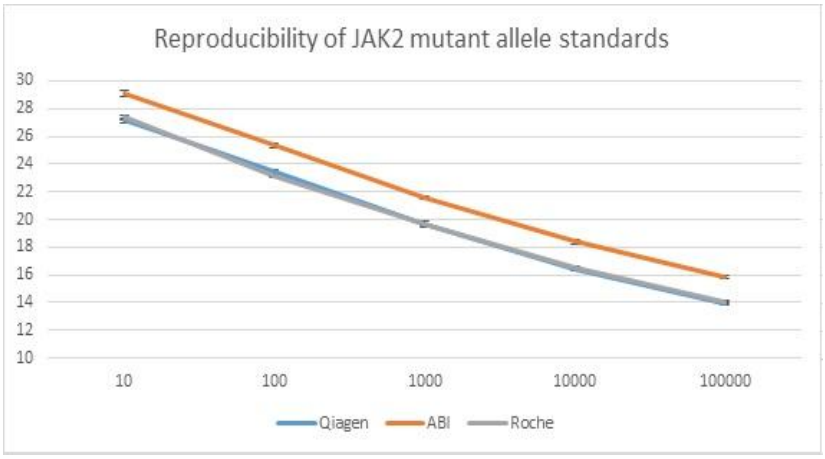

Fig. 3. Reproducibility of *JAK2 V617F* mutant allele standards

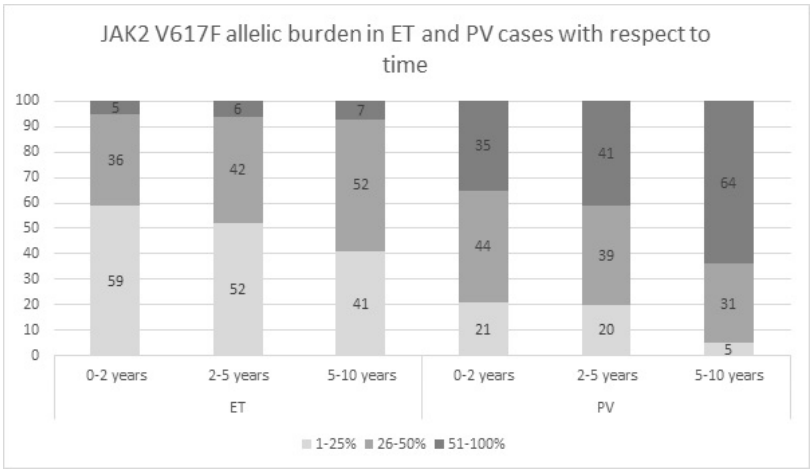

Fig. 4. *JAK2 V617F* allelic burden in ET and PV cases with respect to time

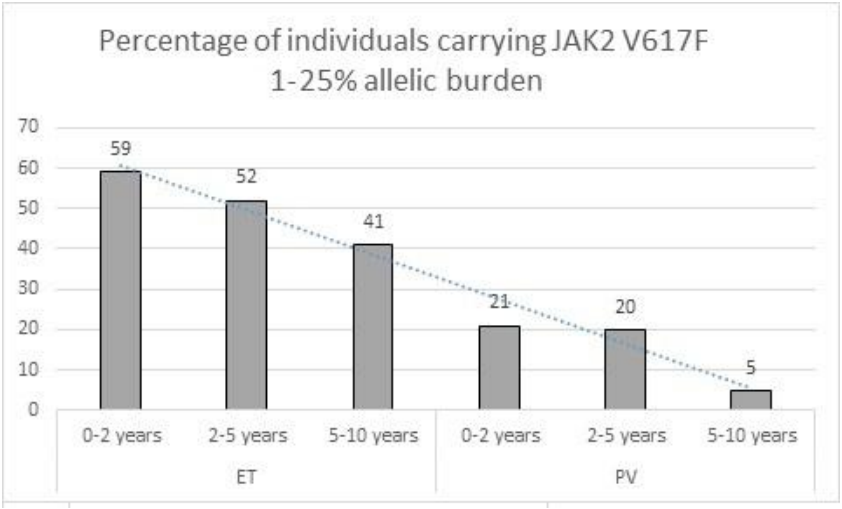

Fig. 5a. Percentage of individuals carrying JAK2 V617F mutation within 1-25% allelic burden range

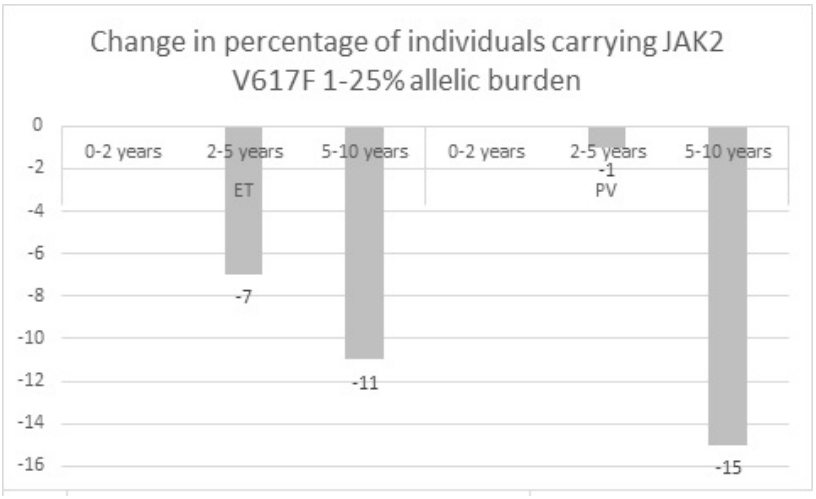

Fig. 5b. Change in percentage of individuals carrying JAK2 V617F mutation within 1-25% allelic burden range

Table 4. Reproducibility of *JAK2 V617F* Mutant allele

Standard (copies/µL)	Cycle threshold (Ct)	Mean	SD	Cycle threshold (Ct)	Mean	SD	Cycle threshold (Ct)	Mean	SD
	Rotorgene			ABI			Roche		
100000	13.8	13.91	0.087	15.72	15.84	0.089	13.96	14.04	0.065
	14.01			15.93			14.06		
	13.94			15.88			14.12		
10000	16.53	16.38	0.108	18.57	18.37	0.145	16.61	16.52	0.065
	16.33			18.22			16.52		
	16.28			18.34			16.45		
1000	19.63	19.63	0.065	21.55	21.55	0.13	19.85	19.66	0.181
	19.55			21.39			19.42		
	19.71			21.71			19.73		
100	23.54	23.51	0.08	25.33	25.34	0.183	23	23.15	0.126
	23.59			25.57			23.31		
	23.4			25.12			23.14		
10	26.96	27.143	0.181	29.01	29.14	0.212	27.33	27.37	0.198
	27.39			29.44			27.64		
	27.08			28.97			27.16		

SD: Standard Deviation

Table 5a. Patients data for ET

	ET (n=167)		
N	**0-2 years**	**3-5 years**	**5-10 years**
	68	**46**	**53**
Sex (male/female)	28/40 (41%/59%)	24/22 (52%/48%)	31/22 (58%/42%)
Age onset (years)	62 (49-67)	63 (52-66)	66 (51-62)
Leucocytes ($X10^9$/L)	10.91 (8.71-15.27)	10.12 (8.03-13.70)	10.68 (7.93-14.87)
Haemoglobin (g/dL)	11.6 (8.07-14.21)	12.09 (9.76-13.61)	12.51 (8.84-14.07)
Platelets ($X10^9$/L)	790 (612-1384)	976 (861-1488)	1018 (721-1354)
BM biopsy Indicative of ET (Yes/No)	67/1 (98.5%)	46/0 (100%)	53/0 (100%)

Table 5b. Patients data for PV

	PV (n=126)		
N	**0-2 years**	**3-5 years**	**5-10 years**
	51	**47**	**28**
Sex (male/female)	23/28 (46%/54%)	24/23 (51%/49%)	16/12 (57%/43%)
Age onset (years)	64 (62-71)	66 (59-70)	66 (62-73)
Leucocytes ($X10^9$/L)	12.3 (11.8-14.2)	15.2 (12.8-16.1)	14.9 (13.4-16.8)
Haemoglobin (g/dL)	17.86 (16.91-19.82)	18.01 (17.01-19.78)	17.32 (16.88-18.12)
Platelets ($X10^9$/L)	591 (493-637)	683 (508-791)	524 (477-692)
Red mass cell >25% above mean normal predicted values	50/1 (98%/2%)	46/1 (98%/2%)	28/0 (100%/0%)

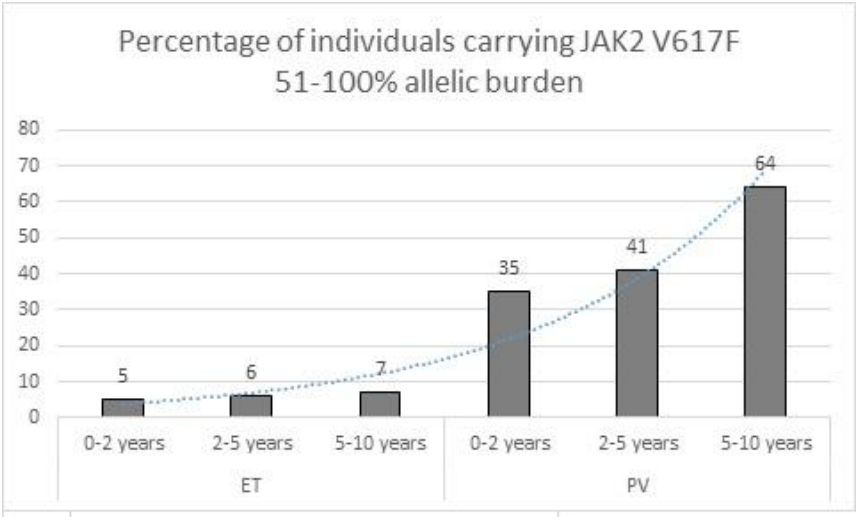

Fig. 6a. Percentage of individuals carrying JAK2 V617F mutation within 51-100% allelic burden range

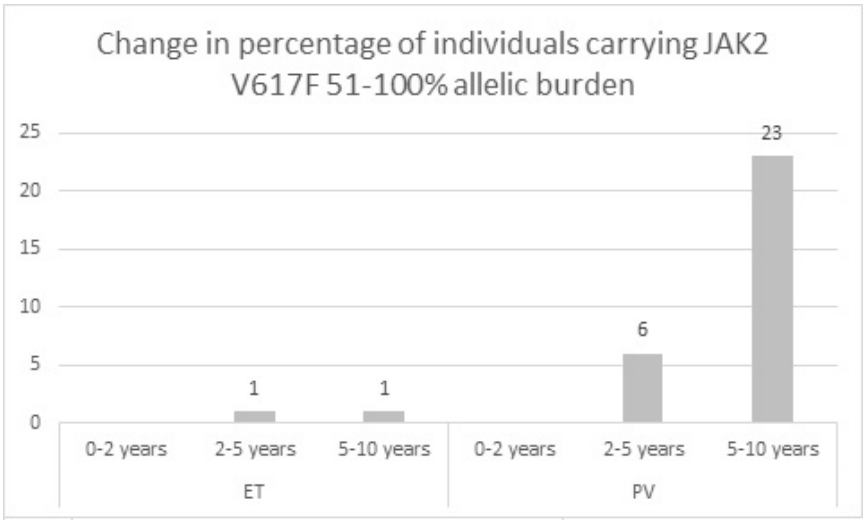

Fig. 6b. Change in percentage of individuals carrying JAK2 V617F mutation within 51-100% allelic burden range

Table 6. Patients distribution for *JAK2 V617F* allelic burden in ET and PV

	ET			PV		
	0-2 years	**2-5 years**	**5-10 years**	**0-2 years**	**2-5 years**	**5-10 years**
1-25%	59	52	41	21	20	5
26-50%	36	42	52	44	39	31
51-100%	5	6	7	35	41	64
	100	100	100	100	100	100

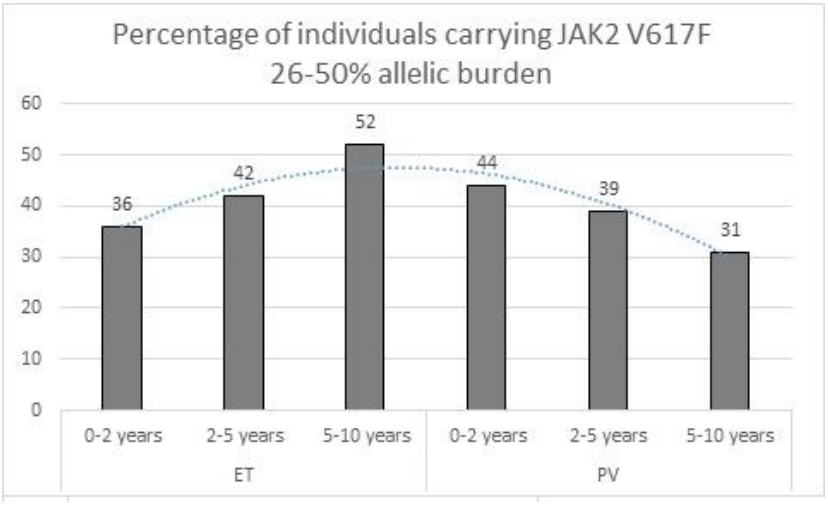

Fig. 7a. Percentage of individuals carrying JAK2 V617F mutation within 26-50% allelic burden range

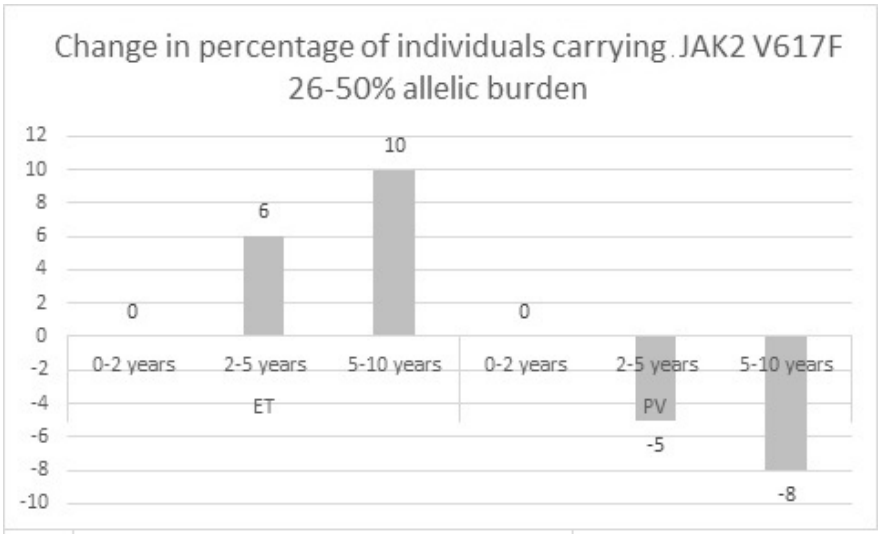

Fig. 7b. Change in percentage of individuals carrying JAK2 V617F mutation within 26-50% allelic burden range

The cost of this gradual reduction in low percentage allelic burden (1-25% category) value across the ET and PV patients can be explained by corresponding trend of increase in >50% allelic burden profile. It showed an increase in percentage value from 5, 6 and 7% in 0-2, 2-5 and 5-10 years respectively in the ET population (Fig. 6a) and a further rise represented by 35, 41

and 64% respectively for the same time blocks in the PV group (Fig. 6a).

The trend of change in allelic burden of the intermediary group of 26-50% *JAK2 V617F* allelic burden showed a bell-shaped curve. While it was seen to gradually increase in the ET population, there was a decrease of % value of this category of allelic burden in the PV group (Fig. 7a and Fig. 7b).

However, careful analysis revealed that this reduction in 26-50% category of *JAK2 V617F* allelic burden in the PV population is expectedly substituted by the high value of >50% allelic burden samples in this sub population (Fig. 6a).

An important observation in this changing trend in allelic burden in the 1-25% (Fig. 5b) and 50-100% (Figure-6b) category in ET and PV patients is rate of change of these values. In the PV population, the fall in value of 1-25% *JAK2 V617F* allelic burden was rather gradual, i.e. a 7% and 11% fall while transiting from (a) 0-2 to 2-5 years and (b) from 2-5 to 5-10 years respectively. The same change in value for the PV patients was steep represented by 1 and 15% for the same time period. Conversely, the rise in the 50-100% allelic burden in the ET population was modest at 1 and 1% for the same time period while it was steep in case of PV group with a rise of 6 and 23% for the same time period respectively.

4. DISCUSSION

Despite MPNs being known to the medical fraternity for a long time, it continues to surprise and confuse clinicians for several reasons. The disease being not widespread, clinicians see lesser patients. This, coupled with the fact that the disease is measured in decades and not days or months, gives lesser chance to the clinicians to measure the disease progression. Still further, the initial symptoms of the MPNs are highly variable and their clinical phenotypes also vary with time. To further complicate the matter, these disorders mimic each other. For example, PV can be misdiagnosed as isolated erythrocytosis [24], leukocytosis [25], thrombocytosis [26,27] or even myelofibrosis [28, 29], whereas isolated thrombocytosis is the key diagnostic feature in around 20% of PMF patients [30]. Further to this, Myelofibrosis is a typical symptom of PV [31,32,33] and erythrocytosis can be seen in PMF during the progression of the disease [34].

Over 40 years from now, MPNs were first demonstrated to be clonal disorders involving a multipotent hematopoietic cell line [35,36]. Since then identification of the *JAK2 V617F* mutation is one of single most important discovery in the advancement of the study of MPNs.

JAK2 is the associated tyrosine kinase of the erythropoietin and thrombopoietin receptors as well as a chaperone involved in their expression on the cell surface [37,38]. The V617F mutation within the JAK2 gene causes permanent activation of this kinase in MPNs [39].

PV, perhaps the most common of all MPNs, has several considerations with its diagnosis, prime amongst which is an activating JAK2 mutation [9, 40]. On the other hand, ET is the only MPN without a clear phenotype, making it not only a diagnosis of exclusion but also one without a single disease entity [41].

It is becoming increasingly evident now that *JAK2 V617F* mutation is one of the single largest factor responsible for the prominent clinical features of MPD. However the impact of this mutation on the phenotype and its progression is dependent on mutant allelic burden of *JAK2 V617F* [42] along with other influences, like the gender of the patient [43]. It is important to note that clonal dominance is time dependent and hence MPDs as a disease is not static and liable to change with time.

In this study, we developed a *JAK2 V617F* quantitation assay kit using Taqman probes and used it to explore the changing trend of this *JAK2 V617F* allelic burden in PV and ET patients over a period of time.

The understanding of the genetic basis of PV, ET and PMF began in the year 2005 with the discovery of *JAK2 V617F* mutation that represented the first recurrent molecular abnormality in BCR-ABL1-negative MPN [8,9,10,11], which was found in more than 95% of patients with PV and around 50–60% in patients with ET or PMF.

Mutations in JAK2 exon 12 and MPL exon 10 were subsequently reported in subsets of patients with *JAK2 V617F*. JAK2 exon 12 mutations have been detected in most of the remaining cases with PV [44], while the MPL exon 10 mutations (mainly involving codon W515) is found in 5–10% of patients with *JAK2 V617F*-negative ET or PMF [45].

In the year 2013, mutations in the CALR gene, were detected in a large proportion of patients suffering from ET and PMF but with a wild-type JAK2 or MPL mutation profile. This CALR gene mutation was a frame shift mutation caused by an exon 9 deletion or insertion namely type-1 (52-bp deletion; p. L367fs*46), and type-2 (5-bp TTGTC insertion; p. K385fs*47) in over 80% of the cases [23,46,47].

This scenario therefore warranted analysis of all the Ph-MPN patients sample used in this study for MPL as well as CALR mutation along with *JAK2 V617F*. The *JAK2 V617F*, CALR exon 9 frame shift mutation, MPL mutation and those which were triple negative occurred at a frequency of 71. 1, 14. 2, 1. 9 and 12. 8% respectively which is broadly in line with other studies [22].

Patients suffering from ET can either harbour the *JAK2 V617F* or bear the wild-type allele [8,10]. Medical observation indicate that patients harbouring the mutation show multiple features that resemble PV [18,48]. This observation has given birth to the contention that probably ET and PV are the ultimate display of the same molecular process where *JAK2 V617F* mutation play a dominant role [49]. Interestingly, the degree of onset of leucocytosis and allelic burden of *JAK2 V617F* mutated allele have been recently linked to the rate of thrombosis both in ET and PV [50] possibly indicating that these two important diseases perhaps reside on the same molecular plane as evident from the progression of vascular complications seen in a patients across the world.

The retrospective analysis and accurate medical record held key to this study. Segregating patients in 3 different time blocks, viz., 0-2, 2-5 and 5-10 years provided an opportunity to trace the trend of variation of the *JAK2 V617F* mutant allele burden with PV and ET population in a single window.

The allelic burden for *JAK2 V617F* was distributed into 3 different loads, viz., 1-25%, 26-50% and those above 50% [51]. Analysis revealed that the two, tail-end burden values (1-25% and >50%) showed a distinct trend of transition with regard to increasing rate of occurrence of the mutant allele burden starting with ET and continuing into PV patients. Fig. 4 shows the *JAK2 V617F* mutant allele burden in 167 ET and 126 PV patients.

Published data indicate that critical vascular episodes by way of major thrombosis in cases of extended survival (>5 years) in ET patients were similar to that of people suffering from PV. This is very similar to BCR/ABL clones in Chronic Myeloid Leukaemia for example, which are seen to expand after a long period of diagnosis [51].

Gale et al. [52] studied the relation between X chromosome inactivation pattern and JAK 2 V617F mutation level in ET patients and demonstrated that the mutant positive clone emained stable for a long period of time [52]. But Tefferi et al. [17] observed a time dependent increase in the *JAK2 V617F* mutant allele burden [17]. Theocharides et al, [53] published an observation spanning across 5 years wherein he observed minor changes in allelic burden for the *JAK2 V617F* mutation burden [53]. While a rising trend in this allelic burden is shown by several authors over a period of time, lack of extended retrospect study, pattern of therapy, study design, length of observational period and number of patients might have contributed to this apparent discrepancies on *JAK2 V617F* allelic burden profile seen by earlier authors.

Retrospective study in large population by earlier authors indicate that the frequency of complications arising out of thrombosis in mutated ET patients is intermediate between ET wild-type and PV in the first 15 years of diagnosis. It was further demonstrated that the rate of severe thrombotic complications progressively increased with increasing allelic burden of *JAK2 V617F* mutant both in ET and PV population with the highest rate seen in the group harbouring >50% *JAK2 V617F* mutant allele burden whi.ch is coincidentally higher in the PV and comparatively lesser in ET [51].

Our retrospective study in Indian population consolidate this observation of increasing *JAK2 V617F* allelic burden trend with time in both ET and PV population. Given the fact that the thrombotic complications are direct function of this allelic burden, the *JAK2 V617F* allelic burden estimation kit developed by our group is of significance in comprehending the expected clinical outcome in ET and PV patients. We have also shown that the rate of change of this allelic burden is much higher in PV group than in ET especially in the post-5 year period thus allowing clinicians to predict the expected thrombotic events that might ensue in a patient based on the

time since he or she was first detected for the disease.

4. CONCLUSION

To the author's knowledge, this is the first Indian study to develop a validated TaqMan chemistry based Real Time PCR *JAK2 V617F* allelic burden estimation kit and use it for a retrospective study to demonstrate that there is a molecular continuation in *JAK2 V617F* mutated ET and PV patients, not only in terms of certain haematological parameters but also at a much deeper molecular level by way of a systematic increase in allelic burden that transverse from ET to PV is a predictable mathematical fashion. More importantly, it correlate to increasingly complicated vascular events. Our study lend credibility to the rationale of estimating *JAK2 V617F* allelic burden rather than simple qualitative determination of the presence or absence of it using allele specific PCR or similar techniques that will only generate limited information with regard to the disease progression in ET and PV patients.

CONSENT AND ETHICAL APPROVAL

As per international standard guideline participant consent and ethical approval has been collected and preserved by the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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