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Clinical and laboratory relevance of JAK2 V617F and BCR-ABL co-existence in Philadelphia positive CML patients

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Abstract: Chronic Myeloid Leukaemia (CML) is characterized by BCR-ABL1 mutation. A number of research studies have published reports of concomitant JAK2-V617F mutation in BCR-ABL positive Chronic Myeloid Leukaemia. This study aims to investigate the frequency of JAK2-V617F mutation in BCR-ABL positive CML cases. After approval from ethical committee, participants were enrolled in the study. A total of 103 samples from CML patients were analysed for the presence of JAK2-V617F mutation using real-time polymerase chain reaction. Patients were monitored for treatment response using real-time quantitative PCR for BCR-ABL1 mutation. Out of 103 samples analysed, 2 patients tested positive for JAK2-V617F mutation. These two patients when treated with standard Tyrosine Kinase Inhibitors (TKI) therapy achieved molecular response and normalized the haemoglobin and white cell counts. However, one patient has sustained thrombocytosis. JAK2 remained positive throughout the treatment course. We could not follow the second patient till the end of the study. JAK2 mutation in BCR-ABL1 mutated CML appears to be rare. Treatment with TKI does not appear to reduce JAK2 mutation burden despite a decrease in BCR-ABL1 copy numbers.

Keywords: JAK2 V617F, BCR-ABL and JAK2, chronic myeloid leukaemia, Ph+ leukaemia, MPN and BCR-ABL.

INTRODUCTION

Myeloproliferative neoplasms (MPN) are complex stem cell-derived neoplasms characterized by abnormal proliferation of myeloid cells. MPN are classified as Philadelphia chromosome-positive Chronic Myeloid Leukaemia (Ph+ CML), polycythemia vera (PV), primary myelofibrosis (PMF), essential thrombocytopenia (ET), and others according to World Health Organization (WHO). These neoplasms can be separated from CML by BCR-ABL transcript negativity (Arber *et al*., 2016). CML is mainly characterized by ABL and BCR translocation producing the Philadelphia chromosome appearing as t(9;22)(q34;q11), a chimeric protein forming 210-*kDa protein* (*p210*) in approximately 90% cases (Arber *et al*., 2016). The Janus Kinase 2 (JAK2) mutation at codon 617 (*JAK2 V617F*), nucleotide G1849T is gain of a function point mutation that causes constitutive tyrosine kinase activity inducing cytokines independent cell proliferation and the auto-inhibitory effect between JAK-homologue (JH)2 and JAK-homologue (JH)1 is disturbed (Tefferi and Pardanani, 2015). JAK2 V617F is mainly confined to PV, PMF, and ET with approximate frequencies of 95%, 60%, and 60% respectively (Tefferi and Pardanani, 2015). BCR-ABL1 and JAK2 V617F were thought to be mutually exclusive until the first report in 2007 (Inami *et al*., 2007). Since then, many case reports (Bader and Dreiling, 2019; Mirza *et al*., 2007; Inami *et al*., 2007; Lorenzo *et al*., 2020; Frikha *et al*., 2021) and studies (Tabassum *et al*., 2014; Azonbakin *et al*., 2018; Soderquist *et al*., 2018; Inami *et al*., 2008; Zaen-Al-Abideen Pahore *et al*., 2011; Aeed *et al*., 2014; AL-Kadmy, 2013; Valikhani *et al*., 2017) have shown the presence of JAK2 V617F in Ph+ CML patients. The frequency of JAK2 V617F in Ph+ CML have been reported from 0% (Azonbakin *et al*., 2018; Inami *et al*., 2008), 0.2% (Martin-Cabrera *et al*., 2017) or 0.4% (Soderquist *et al*., 2018) to 44% (Tabassum *et al*., 2014).

Concomitant JAK2 V617F and BCR-ABL1 patients are reported to have worse prognosis than only BCR-ABL1 positive patients (Lorenzo *et al*., 2020). Ruxolitinib, a JAK1/JAK2 inhibitor, has been approved in Ph negative myelofibrosis. Ruxolitinib has shown a significant effect on the reduction of spleen size in patients, however, the effect on allele burden reduction was not very promising when compared to other treatments (Harrison *et al*., 2012). There has been strong evidence that combining ruxolitinib in combination with tyrosine kinase inhibitors (TKIs) can achieve deep molecular remission in CML. Also, considering JAK/STAT pathway can play role in BCR-ABL independent resistance in CML, the use of JAK2 inhibitors with TKIs has a synergistic effect on lowering BCR-ABL1 transcript levels and overcoming resistance in CML (Sweet *et al*., 2018). These studies reported a positive effect of JAK2 inhibitor when used

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with a TKI in JAK2 negative CML patients. It is hard to evaluate and compare the individual TKI effect versus when used in combination with ruxolitinib from the available data. Use of both the drugs in CML patients with concomitant BCR-ABL and JAK2 V617F have also been reported. TKIs when used in combination with ruxolitinib, helped in relieving symptoms and decrease in spleen size. However, use of TKI only was sufficient to achieve molecular and /or cytogenetic response in these patients (Zhou *et al*., 2015).

The above-mentioned reports suggested that the mutations arose in two different clones. Many postulates have been hypothesized and discussed in the literature about the clonal selection of JAK2 and BCR-ABL1 co-existing in the same patient. To date, it is debatable and unclear to say whether JAK2 and BCR-ABL1 arose in two different clones, a single clone with one disease being dominant and suppresses the other. These speculations are mainly made based on the increase or decrease in the mutation burden of one of the two concomitant mutations. It is still unclear whether the JAK2 mutation occurs first (Mirza *et al*., 2007) or is expressed after treatment of Ph+ patients (Inami *et al*., 2007). In recent years, intensive research revealed that increase JAK2 allele burden is associated with aggressive phenotype and disease severity in Ph negative MPN. Hydroxyurea has been studied extensively for its effect on decreasing the JAK2 allele burden. It can lower the percentage of the JAK2 allele but statistically significant reduction has not been observed by all researchers (Antonioli *et al*., 2010).

Considering the sparsity of data and the variations in the reported frequencies of JAK2 V617F in Ph+ CML patients, we set-up a prospective study. We asked, what is the frequency of JAK2 and BCR-ABL1 concomitant presence in Ph+ CML patients? Whether JAK2 expressions can be masked or under-expressed when hydroxyurea is used for short-term or TKIs for long-term? Clonal selection, based on Minimal Residual Disease (MRD) monitoring or cycle threshold (Ct) and prognostic impact of TKIs when both the mutations co-existed.

MATERIALS AND METHODS

Study design and patients

This research was part of a larger prospective cohort study designed to explore resistance mechanisms in CML patients. Patients were enrolled after cytogenetic or molecular evidence for the Philadelphia chromosome. Patients were enrolled in early 2017 till 2019 after informed consent and 5-10 ml blood was collected by venipuncture before the start of any TKI. 103 patients could be assessed for screening JAK2 mutation. The patients were followed up during their visit to the oncology ward, "MTI Hayatabad Medical Complex" (HMC) hospital, Peshawar (Fig. 1). Ethical approval was granted by "Khyber Medical University Ethical Committee". The primary goal was to test MRD for BCR-ABL1. However, the presence of JAK2 V617F and other clinical features were followed secondary to BCR-ABL1 quantification.

RNA extraction

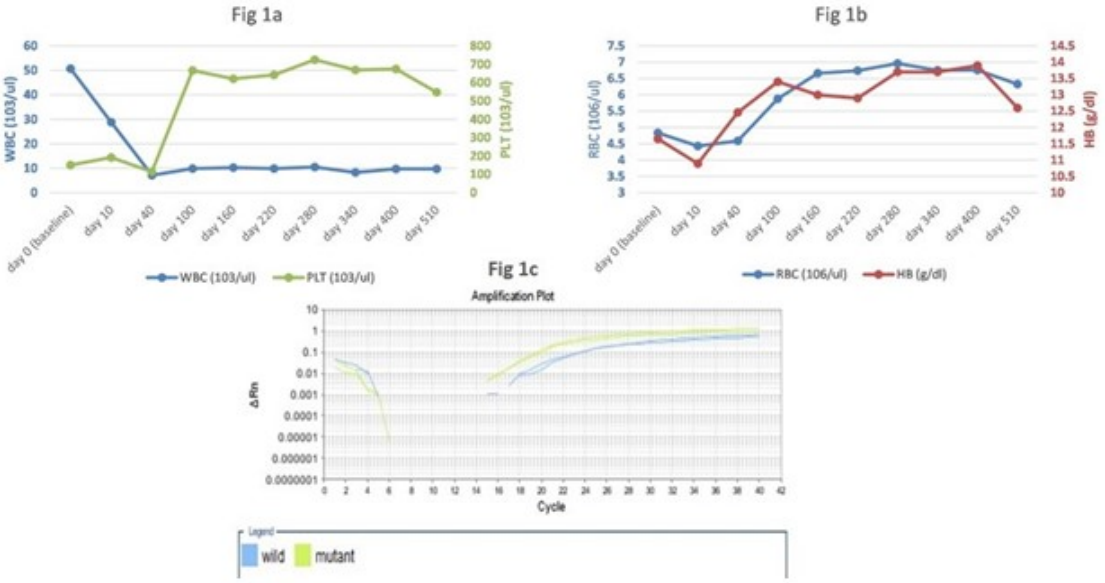

Fig. 1: Clinical and laboratory parameters at diagnosis and during various time-points after initiating TKI therapy
Fig. 1a: WBC (blue, left y-axis) and platelets (green, right y-axis) plotted against various time points (x-axis) to show changes during treatment with TKIs
Fig. 1b: RBC (blue, left y-axis) and haemoglobin (red, right y-axis) plotted against various time points (x-axis) to show changes during treatment with TKIs
Fig. 1c: Allele-specific amplification plot of JAK2 V617F wild (blue) and the mutant (green) allele for patient1

Table 1: Pre-TKI (Baseline) and Post-TKI

	Variables	Mean ± Std. Dev.	95% CI	Minimum	Maximum
Pre-TKI	WBC (10^3/µl)	181.13±123.5	157.07 to 205.36	17	660
	Hb (g/dl)	10.23±1.9	9.8 to 10.6	5	14.3
	PLT (10^3/µl)	385.5±251.2	336.4 to 434.6	39	1326
Post-TKI	WBC (10^3/µl)	6.8±4.09	6 to 7.6	1.32	40.5
	Hb (g/dl)	11.3±1.9	10.9 to 11.6	6	15.1
	PLT (10^3/µl)	282.3±205.1	242.25 to 322.4	22	1142

Table 2: Percentages of the JAK2 mutation, splenomegaly, hepatomegaly, MMR and use of Hydroxyurea

N = 103			
Variables	Yes n (%)	No n (%)	NA (%)
JAK2 V617F	2 (1.94)	101 (98.06)	0
Hepatomegaly	32 (31)	61 (59.22)	10 (9.7)
Splenomegaly	84 (81.5)	9 (8.74)	10 (9.7)
MMR=MR^3*	44 (42.72)	59 (57.28)	0
Hydroxyurea	74 (71.8)	26 (25.24)	3 (2.9)

*MR^3; BCR-ABL1 less than or equal to 0.1% after 12 months

Table 3: Characteristics of JAK2 positive patients

	Use of TKIs	WBC 10^3/µl	RBC 10^6/µl	Hb g/dl	PLT 10^3/µl	Spleno megaly	Hepato megaly	Blast %	Cytog enetic	FISH# %	Glucose mg/dl	LDH U/L	JAK 2
Patient 1	Before TKI	50.7	4.83	11.6	971	Yes	Yes	7	20/20	98	499	612	Pos
	After 6 months	10.25	6.64	13	666	NA	NA	NA	NA	8	NA	NA	NA
	After 12 months	9.7	6.33	12.6	621	NA	NA	NA	NA	ND!	NA	NA	Pos
Patient 2	Before TKI	85	5.3	13	519	YES	No	<5	20/20	98	120	250	Pos
	After 6 months	9.9	4.8	12.5	204	NA	NA	NA	NA	2	NA	NA	NA
	After 12 months	7.6	3.7	12	326	NA	NA	NA	NA	ND	NA	NA	Pos

For patient 1 and patient 2, PCR results are written instead of FISH after 12 months, ! ND means not detected/ achieve MMR (BCR-ABL1 <0.1%). NA mean not available and/or not applicable

RNA was extracted using Trizol reagent with slight modifications (Toni *et al*., 2018), subsequently synthesizing cDNA using random hexamer and Revert Aid M-MuLV RT (Revert Aid First Strand cDNA Synthesis Kit, Thermo Fisher Scientific; catalog no K1622). The RNA (one-step PCR) or cDNA (two steps PCR) was used for BCR-ABL1 quantification or stored at -80°C. The Ph+ chromosome and/ or BCR-ABL1 transcript analysis was performed by cytogenetic, reverse transcription-quantitative polymerase chain reaction (qRT-PCR) and fluorescent in-situ hybridization according to the standard protocols.

DNA extraction

DNA was extracted using 'GeneJET Genomic DNA Purification Kit' (Thermo Fisher Scientific; catalog no K0722) according to the manufacturer's instructions. The DNA concentration was quantified using a spectrophotometer and concentration was normalized for each sample to be used for JAK2 analysis or stored until further use at recommended storage conditions.

Quantitative Real-time PCR for BCR-ABL1 mutation

Real-time quantitative PCR for BCR-ABL mutation was performed using "BCR-ABL P210 (Mbcr) One-Step Detection Kit" (EntroGen; cat no BCR-210 QRT46) that amplified p210 b2a2 and b3a2 transcripts on International Scale (IS). Also, we optimized in-house method in parallel. Briefly, primers and probes from "Europe Against Cancer" (EAC) consensus for ABL and Mbcr (Beillard *et al*., 2003; Gabert *et al*., 2003) were synthesized by "Eurofins Genomics" and used with "Taq Probe 2X qPCR Master Mix-Low ROX" (abm Canada; cat. no Mastermix-PL).

Primer and probe concentrations were used as per EAC guidelines and default thermal cycling conditions on ABI 7500 (Thermo Fisher Scientific) standard platform were used. According to the manufacturer's protocol (EntroGen), 40-120ng RNA for one-step or RNA equivalent to yield approximately 100ng/5ul for cDNA synthesis was added into the master mix. Four dilutions for ABL (20000 to 224 copies/ul), Mbcr (3600 to 24

copies/ul), positive control and negative control were provided with the kit to calculate absolute copy number according to IS ratio for Mbcr. Positive controls, negative controls and no-template controls were used in all assays. PCR data were analysed using ABI 7500 Software v2.3. qPCR was performed every 3-4 months to assess MRD for BCR-ABL1 (Baccarani *et al*., 2013).

Real-time PCR for JAK2-V617F mutation

The JAK2 V617F mutation analysis was performed using allele-specific real-time PCR (Jovanovic *et al*., 2013). The ELN/COST JAK2 specified a common forward primer, allele-specific reverse primers and a FAM labeled probe. The primers and probe were obtained from "Eurofins Genomics". Initially, primers were confirmed against JAK2 V617F positive samples and healthy controls on an agarose gel.

The set of primers and probes have a proven sensitivity and specificity in comparison to other assays. 25ng DNA was used per reaction with master mix, PCR platform, analysis software and cycling conditions same as above. To further validate the results, twenty patients were tested at Agha Khan University Hospital Karachi (AKUH) for JAK2 V617F mutation analysis.

STATISTICAL ANALYSIS

The mean, standard deviation and confidence interval were calculated using Stata version 12. The frequency was low and hence no prognostic comparison could be performed between JAK2 positive and JAK2 negative CML patients.

RESULTS

Clinical and laboratory findings

All the 103 patients were Ph+ CML confirmed through bone marrow analysis. At the time of recruitment, two patients were in the accelerated phase, one in blast crises and one progresses to the advance phase later according to the ELN guidelines (Baccarani *et al*., 2013). On day 0 (before the start of TKIs), the mean age was 41.8±15.78 (range 7 to 75 years), WBC ranged from 17 to 660 10^3/µl (181.13±123.5). The haemoglobin and platelets ranged from 5 to 14.3g/dl (10.23±1.9) and 39 to 1326 10^3/µl (385.5±251.2) respectively as shown in Table 1. The pre and post-TKIs blood profiles after 12 months are shown in Table 1.

Our cohort had a male predominance of 60% to 40% female for CML. Splenomegaly and hepatomegaly or abnormal spleen and or liver were found in 81.5% and 31 % of patients respectively. Most (71.8%) of the suspected CML patients were prescribed hydroxyurea until the confirmation of Ph+ chromosome in bone marrow metaphase by cytogenetic reports. They were prescribed TKIs once confirmed. After 12 months follow up, 61.2% were using Imatinib and 38.8% were on Nilotinib currently and 42.7% had achieved MR^3 (table 2).

Co-existing BCR-ABL1 and JAK2-V617F mutation

The JAK2 V617F and BCR-ABL1 p210 mutations were present concomitantly in 2 (1.94%) patients. Both the JAK2 V617F positive patients were presented in extremely lethargic conditions. Patient 1 (Table 3) was showing a good response initially. Later the platelets and RBCs kept on raising (Fig. 1a). Initial bone marrow aspirate showed myeloid hyperplasia with an increase in myelocytes, metamyelocytes and promyelocytes for both of the patients. We did not see any significant variations in the response of the patients except the greater variation in platelet count for patient 1. Both the patient's Hb, leukocytes and BCR-ABL1 were well comparable to other patients (Fig. 1b). Many of the JAK2 negative patients had a worse prognosis than the JAK2 positive Ph+ CML patients including transformation to the advanced phases of CML. The Ct values in Fig. 1c were taken before the start of TKI and after using hydroxyurea for a month. Almost the same Cts were achieved when tested and amplified after MR^3.

DISCUSSION

In the recent past, many studies on CML with aberrant and co-existing genetic variation were performed. Many case reports and studies have reported the JAK2 V617F and BCR-ABL co-existence in CML. Comparatively, larger prospective studies still lack to elaborate the prognosis of the JAK2 V617F and BCR-ABL co-existence in CML. Our study investigated the presence of JAK2 V617F mutation in 103 Ph+ CML patients prospectively enrolled to study resistance mechanisms in CML (to be published elsewhere). We found 2 (1.94%) patients with JAK2 and BCR-ABL1 occurring simultaneously.

The study aimed to confirm the results of published Asian and non-Asian populations studies (Zaen-Al-Abideen Pahore *et al*., 2011; Tabassum *et al*., 2014; Aeed *et al*., 2014; AL-Kadmy, 2013; Valikhani *et al*., 2017). Two reports (Zaen-Al-Abideen Pahore *et al*., 2011; Tabassum *et al*., 2014) from Pakistan and one other (Aeed *et al*., 2014) from Sudan have reported 26.7%, 44% and 38.8% of JAK2 V617F mutation respectively in CML patients. Our results and two other reports from Iran and Iraq disagree with such high incidence by reporting 1.94%, 6% and 1.1% respectively (Valikhani *et al*., 2017; AL-Kadmy, 2013). Others have reported low JAK2 frequency; 0% (Azonbakin *et al*., 2018; Inami *et al*., 2008) or 0.2% (Martin-Cabrera *et al*., 2017) or 0.4% (Soderquist *et al*., 2018), which reflects our findings. A retrospective study of 592 CML patients reported 0.68% of JAK2 mutations (Lewandowski *et al*., 2018). In a larger retrospective

Table 4: Comparison of methodologies

S. No.	Study (population)	Methodology/ Principal	Sensitivity	JAK2 detected/ total tested
1	Pahore et al (Pakistan) (Zaen-Al-Abideen Pahore et al., 2011)	Nested PCR/ gel electrophoresis	Less sensitive than RQ allele-specific PCR; many steps PCR can increase chances of contamination. Also, no reconfirmation was performed	12/45
2	Tabassum et al (Pakistan) (Tabassum et al., 2014)	Seeplex kit, Korea/ Allele-specific/ gel electrophoresis	Cannot find relevant information. Also, no reconfirmation	11/25
3	Aeed et al (Sudan) (Aeed et al., 2014)	Allele-specific competitive blocker/ Allele-specific PCR	Less sensitive than RQ allele-specific PCR; No confirmation	19/49
4	Valikhani et al (Iran) (Valikhani et al., 2017)	ARMS PCR/ Allele-Specific and sequencing	Less sensitive than RQ allele-specific PCR; Confirmation by sequencing	4/66
5	Lewandowski et al (Poland) (Lewandowski et al., 2018)	RT-qPCR	ELN consensus primers/ assay	4/592
6	Soderquist et al United States) (Soderquist et al., 2018)	Allele discrimination assay or sequencing	Real-time PCR; less contamination	6/ 1570*
7	Azonbakin et al (Benin) (Azonbakin et al., 2018)	ARMS-PCR/ Gel-based	Less sensitive than RQ allele-specific PCR; gel image is clear, no reconfirmation	0/27
8	Inami et al (Japan) (Inami et al., 2008)	Allele-Specific PCR and sequencing	Less sensitive than RQ allele-specific PCR; confirmed by sequencing	0/72
9	Al-Kaabi et al (Iraq) (AL-Kadmy, 2013)	ARMS-PCR/ gel-based	Less sensitive than RQ allele-specific PCR; gel image is clear, no reconfirmation	1/89
10	Ours (Pakistan)	Allele-Specific RT-qPCR	More sensitive/ confirmation from another lab	2/103

study, the estimated frequency was 0.4% for JAK2 and BCR-ABL co-existence in 1570 suspected MPN individuals (Soderquist *et al*., 2018). We showed that 44, 38.8, or even 26.7% frequency is too high and larger prospective cohort studies are required using more sensitive techniques. We did not identify the possible reason but the only explanation could be the wide variation amongst the different methodologies across the literature. Table 4 indicates several assays performed to detect the mutation. We followed the standard UK guidelines for sample collection and processing (Bench *et al*., 2013). According to these guidelines, the two most sensitive methods for JAK2 V617F detection are ARMS/ Allele-specific PCR and real time allele-specific PCR with sensitivity of 0.1-5% and 0.01-1%. This indicates that real-time allele-specific PCR is the most sensitive with lesser chances of contamination (Bench *et al*., 2013).

Of both the JAK2 positive patients, one was male and the other was female, both were using nilotinib and were presented with weakness and body pain. The initial data for one patient (patient 2) could not be retrieved as he got initial symptomatic treatment (not TKIs) elsewhere, however, his JAK2 V617F was still detectable before and after the use of TKIs. Whereas, the other patient had used hydroxyurea for a month when tested for JAK2 V617F and again after 18 months of using nilotinib, both of the times JAK2 was positive. We observed that both the patients achieved a molecular response, though complete haematologic response could not be achieved (Lorenzo *et al*., 2020). Based on the available data, it is obvious that neither hydroxyurea (short-term) nor nilotinib suppresses JAK2 mutation to undetectable levels (Antonioli *et al*., 2010; Kim *et al*., 2020). In unpublished data of BCR-ABL1 -negative MPN, we were unable to find a significant reduction in JAK2 allele burden after long-term hydroxyurea. Ruxolitinib, a JAK2/JAK1 inhibitor, has been reported to have an additive effect in relieving symptoms in CML patients when used in combination with TKIs (Zhou *et al*., 2015). It is uncertain whether this combination therapy can overcome resistance. Until prospective clinical trials are conducted, this decision is based on clinical judgement and local clinical guidelines.

Our findings raise some important questions. Whether JAK2 precedes BCR-ABL1 or vice versa and if they occur in the same clone or occur mutually exclusive? A recent article has described 34 (including itself) case

reports where 15 cases showed concomitant CML plus JAK2 and were classified to have one of the three myeloproliferative disorders (MPDs), 10 cases diagnosed as MPDs and then BCR-ABL1 and in 9 cases first BCR-ABL1 was detected (Bader and Dreiling, 2019). We showed that both mutations co-existed in both of the patients. Many hypotheses have been presented in the case reports explaining the clonal selection. In our study, JAK2 was constant while BCR-ABL1 was decreasing. We could not perform allele burden for JAK2 but based on Ct values, allele burden was constant even after a year or longer treatment with a TKI. Based on the data we have, it can be hypothesized that both the patient's mutation occurred either in two individual clones or JAK2 clone with a BCR-ABL1 subclone as suggested (Bader and Dreiling, 2019). In concordance with other reports (Zhou *et al*., 2015), we believe that TKIs do not affect JAK2 expression if existed concomitantly with BCR-ABL1. Both patients were presented with CML phenotype at the time of diagnosis and hence treated as CML. Further, both of the patients were not asked for JAK2 mutation analysis as part of their routine laboratory diagnosis. No additional treatment was given for JAK2 mutation by the treating physicians.

CONCLUSION

The concomitant presence of JAK2 V617F and BCR-ABL in CML patients is a rare phenomenon. Also, neither nilotinib nor hydroxyurea lowered the JAK2 to undetectable levels.

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