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Original Article

Prevalence of JAK2^{V617F} Mutation and Serum Levels of Alkaline Phosphatase and Lactate Dehydrogenase in Chronic Myelogenous Leukemia

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ABSTRACT

Chronic myelogenous leukemia (CML) is a myeloproliferative neoplasm arises from Bcr-Abl gene translocation (called Ph chromosome) in hematopoietic stem cells (HSCs). JAK2^{V617F} mutation is an acquired single nucleotide polymorphism (SNP) occurs in JAK2 gene and is associated with many hematological malignancy other than CML. This study aimed to investigate the prevalence of JAK2V617F mutation and serum levels of alkaline phophatase (ALP) and lactate dehydrogenase (LDH) in Ph+ CML Iraqi patients treated with imatinib. Blood samples were collected from 42 Ph+ CML patients who have been received at least six month therapy with imatinib. DNA was extracted, and real time polymerase chain reaction (qPCR) was used for JAK2^{V617F} detection. Serum levels of ALP and LDH were measured using ready kits. Five of 43 CML patients (11.62%) had heterozygous mutant allele of JAK2^{V617F} mutation, with a concentration ranged from 0.01% to 0.12%. The prevalence of this mutation is more associated with male than female (OR=0.5, 95%CI=0.364-0.687). JAK2^{V617F}-positive patients had higher average serum levels of ALP and LDH (146.05±8.028 IU/L and 204± 10.85 IU/L respectively) than that of JAK2^{V617F}-negative patients (64.45±40.15 IU/L and 178.33±13.693 IU/L respectively with significant differences. JAK2^{V617F} mutationcould occur in coexistence with Bcr-Abl transcript in CML patients, and serum levels of ALP and LDH can be used as indicators for this coexistence.

Keywords: CML, Bcr-Abl, JAK2^{V617F} mutation, ALP, LDH, qPCR

INTRODUCTION

Chronic myelogenous leukemia is a myeloproliferative neoplasm arises from a single genetic translocation in a pluripotential HSC. This genetic aberration results in a clonal overproduction of the myeloid cell line with a preponderance of immature cells in the neutrophilic line [1]. A hallmark of CML is an abnormal chromosome known as the Philadelphia Chromosome, a genetic or DNA related abnormality that initiates a series of events leading to the development of what is called a Bcr-Abl translocation. This translocation results in an abnormality of an enzyme known as tyrosine kinase causing granulocytes to grow and reproduce rapidly[2]. There is no obvious reason associated with such Ph Chromosome, but the disease is seen predominantly in those aged 46 to 53 years, and is slightly more common in males than in females [1]. Exposure to ionizing radiation and prolonged used of chemotherapy have also been committed as risk factor for the disease[3]. Tyrosine kinases inhibitors have become the standard drugs which are successfully used for the treatment of CML; however, in some cases, CML develops resistance to therapy with such drugs. JAK2^{V617F} mutation can be a candidate to affect the overall status of CML patients including the possibility of resistance to tyrosine kinase inhibitor drugs. Although Ph+-CML patients formerly regarded as lacking this mutation, growing number of case reports and prevalence studies have refuted this claim and confirmed the coexistence of both genetic abnormalities in the same patients [4,5,6,7]. The present study was conducted using qPCR to investigate the prevalence of JAK2^{V617F} mutation and serum levels of ALP and LHD among Ph+-CML patients.

SUBJECT AND METHOD

A total of 43 patients (25 Male and 18 female; age range 16-80 years) with Ph+CML attending Iraqi

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National Center of Hematology for Research and Treatment/ Baghdad from September 2013 to January 2014 were enrolled in this study. Ethical clearance was sought and obtained from this center. Data were collected through direct interview with the patient, and by seeking his/her hospital record as well as previous medical reports Patient's claims were followed as an alternative source of information when his/her previous medical reports were not available. Informed consent form was obtained from each patient. This form included age, sex, height, weight previous and current occupation, smoking, drinking, residence, and first relative family history of leukemia.

Blood samples: Five-milliliter of blood was taken from each patients. Granulocyte was separated and DNA was extracted using ready kit (gSYNCTM DNA Mini Kit Whole Blood Protocol/ Geneaid/ Korea) Patients 'sera were screened for the serum level of ALP and LDH. qPCR reactions on Exicycler™ 96 Real Time Quantitative Thermal Block/Bioneer/ South Korea. The primers used for qPCR were: forward primer: 5'-AAG CTT TCT CAC AAG CAT TTG GTT T-3'; mutation-specific reverse primer: 5'-AGA AAG GCA TTA GAA AGC CTG TAGTT-3'. Taqman probe: 5'-6-FAM-TCCACAGAAACATAC-MGB-BHQ-3'.

The qPCR was achieved in 50 µl reaction where the concentration of each primer was 300nM, and the concentration of the probe was 200 nM. In order to determine the sensitivity of the mutation-specific primer set, a standard curve was created by 5-fold dilution series of homozygous JAK ^{V617F} mutated DNA initially taken from patients with more than 90% mutated alleles (Figure 1).

Alkaline Phosphatase and Lactate Dehydrogenase: A commercial kit (SyrBio/Syria and Randox Laboratory Limited/ UK) were used to estimate ALP and LDH respectively activity in the serum samples.

Statistical Analysis: The Statistical Package for the Social sciences (SPSS, version 14) was used for statistical analysis. Risk association between the JAK2^{V617F} mutation and each of risk factor was estimated by the calculation of adjusted odd ratio and 95% confidence intervals using logistic regression. Independent t-test was used to compare means of WBC count, serum levels of ALP and LDH. A *p*-value < 0.05was considered statistically significant.

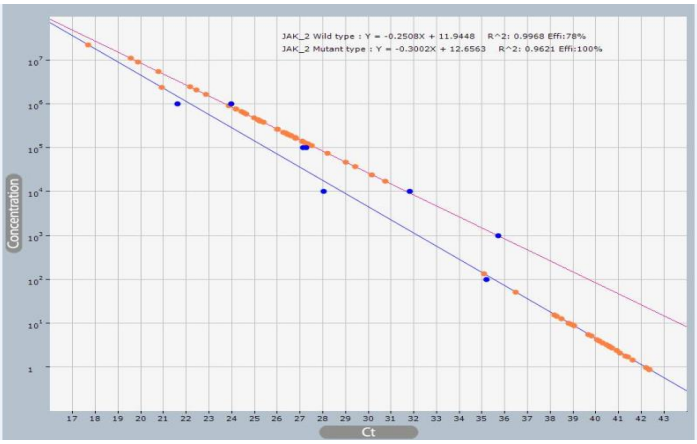

Figure 1: Standard curve of dilution homozygous JAK^{V617F} mutated D using qPCR.

RESULTS

Real Time PCR (qPCR): Real Time PCR was used to investigate the occurrence of JAK2V617F mutation among Ph+-CML patient. Taqman probe with FAM and BHQ as fluorophoreand quencher respectively was employed for the detection of PCR products.(Figure 2).

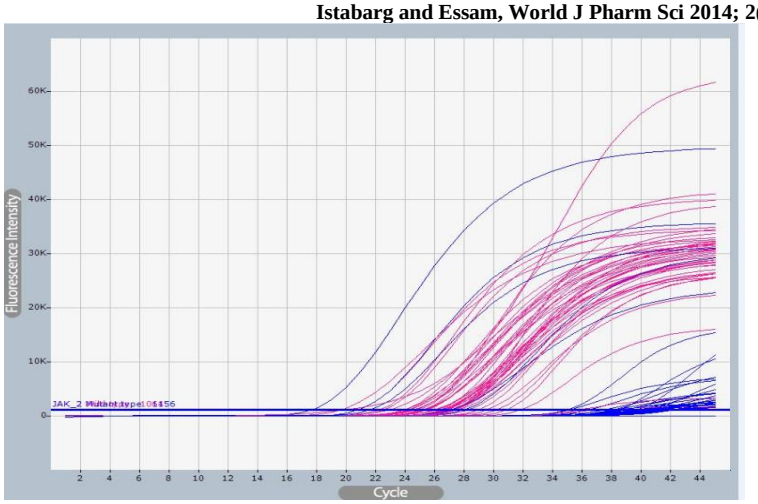

Figure 2: The real time PCR progression curve for JAK2^{V617F} mutation samples.

As JAK2^{V617F} mutation has two allele: mutant (T) and wild(G), there were two separated qPCR results each for eitheralleles. For the mutant all (table 1), the result revealed that 5 from 43 Ph+-CML patients (11.62%) gave positive results for this allele. On the other hands all patients gave positive result for the wild type allele (Table 2). That means all the 5 patients with positive result for mutant JAK2V617Fare heterozygous for this mutation (the genotype is GT). Not only qPCR determines the genotype of the mutant allele, but also the quantity of the mutant as well as the wild type allele. Table 3 shows the quantities of mutant allele in the five positive samples for this allele. Although the quantities were very small (ranged from 0.01% to 0.12%), they were detected by qPCR and recorded as positive results.

Table 1:Part of qPCR result of the mutant allele showing the five positive samples (A8,F8, G8,C9 and D9).

Well	Sample ID	CT	Result	JAK_2 Mutant ty	JAK_2 Mutant	JAK_2 Mutant	JAK_2 Mutant	Result
C1	MTPC	0	Valid	40.47	3.87E+00	1.94E+02	1.94E+02	Negative
A7	NTC	0	Valid	Undetermined	-	-	-	Valid
B7	MTPC	0	Valid	16.51	1.68E+07	8.41E+08	8.41E+08	Positive
D7	STD1	0	Valid	35.01	1.00E+02	-	-	Valid
E7	STD2	0	Valid	32.32	1.00E+03	-	-	Valid
F7	STD3	0	Valid	28.21	1.00E+04	-	-	Valid
H7	STD5	0	Valid	21.26	1.00E+06	-	-	Valid
A8	Sample01	0	Valid	36.73	4.21E+01	2.10E+03	2.10E+03	Positive
B8	Sample02	0	Valid	39.62	6.66E+00	3.33E+02	3.33E+02	Negative
F8	Sample06	0	Valid	35.51	9.16E+01	4.58E+03	4.58E+03	Positive
G8	Sample07	0	Valid	35.45	9.52E+01	4.76E+03	4.76E+03	Positive
H8	Sample08	0	Valid	41.14	2.52E+00	1.26E+02	1.26E+02	Negative
C9	Sample11	0	Valid	37	3.54E+01	1.77E+03	1.77E+03	Positive
D9	Sample12	0	Valid	36.01	6.66E+01	3.33E+03	3.33E+03	Positive
E9	Sample13	0	Valid	41.79	1.67E+00	8.34E+01	8.34E+01	Negative
H9	Sample16	0	Valid	39.97	5.32E+00	2.66E+02	2.66E+02	Negative

Table 2: Part of qPCR result of the wild type allele. All samples gave positive results.

Well	Sample ID	CT	Result	JAK_2 Wilc	JAK_2 Wilc	JAK_2 Wilc	JAK_2 Wilc	Result
A1	NTC	0	Valid	Undeterm	-	-	-	Valid
B1	WTPC	0	Valid	20.77	5.45E+06	5.45E+08	5.45E+08	Positive
D1	STD1	0	Valid	36.91	1.00E+02	-	-	Invalid
E1	STD2	0	Valid	35.7	1.00E+03	-	-	Valid
F1	STD3	0	Valid	31.81	1.00E+04	-	-	Valid
G1	STD4	0	Valid	27.28	1.00E+05	-	-	Valid
H1	STD5	0	Valid	23.96	1.00E+06	-	-	Valid
A2	Sample01	0	Valid	27.34	1.23E+05	1.23E+07	1.23E+07	Positive
B2	Sample02	0	Valid	22.16	2.44E+06	2.44E+08	2.44E+08	Positive
C2	Sample03	0	Valid	26.45	2.05E+05	2.05E+07	2.05E+07	Positive
D2	Sample04	0	Valid	25.24	4.12E+05	4.12E+07	4.12E+07	Positive
E2	Sample05	0	Valid	27.21	1.32E+05	1.32E+07	1.32E+07	Positive
F2	Sample06	0	Valid	29.43	3.67E+04	3.67E+06	3.67E+06	Positive
G2	Sample07	0	Valid	26.8	1.68E+05	1.68E+07	1.68E+07	Positive
H2	Sample08	0	Valid	19.88	9.11E+06	9.11E+08	9.11E+08	Positive
A3	Sample09	0	Valid	24.99	4.76E+05	4.76E+07	4.76E+07	Positive

Table 3: qPCR final results showing the quantities of the five positive samples for mutant JAK2^{V617F} allele.

Sample	Wild type Concentration	Mutant type Concentration	Total Concentration	Counted Mutant ratio(%
Wild Control	23805400.14	3.87	0	0
Mutant Control	0	16828006.19	1	100
Sample01	1496068.15	42.07	0	0
Sample02	579552.63	6.66	0	0
Sample03	464708.51	0	0	0
Sample04	283653.75	0	0	0
Sample05	296848.72	0	0	0
Sample06	76873.71	91.62	0	0.12
Sample07	438320.81	95.19	0	0.02
Sample08	87538.04	2.52	0	0
Sample09	17145.05	0	0	0
Sample10	60450.9	0	0	0
Sample11	38116.71	35.41	0	0.09
Sample12	203666.76	66.6	0	0.03
Sample13	35952.32	1.67	0	0
Sample14	57763.85	0	0	0
Sample15	65777.16	0	0	0
Sample16	557400.29	5.32	0	0
Sample17	65777.16	0	0	0
Sample18	40411.41	0	0	0
Sample19	9250.15	0	0	0
Sample20	14293.95	1.52	0	0.01

Risk factors: The initial consent involved drinking, family history and occupation beside the age, gender, weight, height, smoking status and residence. For drinking, none of the participant mentioned that he or she used to drink formerly or at present. All the patients, except one who has his grandmother with breast cancer, have no family history of cancer. There were great diversities in occupations which give no sense in statistical analysis. Therefore, these risk factors have been dropped from the analysis which is confined to age, gender, BMI, smoking and residence (Table 4). JAK2^{V617F}-positive group had higher mean age (49.2±16.24 years) than JAK2^{V617F}-negative group (38.71±14.87 years); however, the difference was insignificant (p= 0.162, OR= 1.052, 95%CI= 0.980- 1.128). All patient positive for JAK2^{V617F}mutation were male, while exactly half patients with JAK2^{V617F}-negative were males with significant difference (p= 0.034, OR= 0.5, 95%CI=

0.364-0.687). Height and weight were used to calculate BMI according to the fomula: BMI= weight (Kg)/(height (m))^2. Mean BMIs were very closed in the two groups (26.82 ±3.81 and 25.92±5.44 in JAK2^{V617F}-positive and negative respectively) and no significant difference was recorded (p= 0.716, OR=1.032, 95%CI=0.782 - 1.22). Because the relative low numbers of smokers among participant, smoking status categorized into only two categories: smokers which involved ex- and/or current smokers and those who never smoked. Statistical test revealed no significant differences in the percentages of smokers between JAK2^{V617F} positive group (20%) and JAK2^{V617F}-negative group (7.89%) (p= 0.318, OR= 2.917, 95%CI= 0.24-35.122).

Table 4: Association of some risk factors with the occurrence of JAK2^{V617F}mutation.

Risk factors	JAK2^{V617F} N=5	CML N=38	P-value	OR(95%CI)
Mean age in years (SD)	38.71 (14.87)	49.2 (16.24)	0.162	1.052 (0.980- 1.128)
Gender Male Female	5 (100%) 0 (0%)	19 (50%) 19 (50%)	0.034	1.0 0.5 (0.364-0.687)
Mean BMI (SD)	26.82 (3.81)	25.92 (5.44)	0.716	1.032 (0.872-1.22)
Smoking Never Smoker	4(80%) 1 (20%)	35 (92.1%) 3(7.89%)	0.318	1.0 2.917 (0.24-35.122)
Residency Rural Urban	1 (20%) 4 (80%)	10 (26.32%) 28 (73.68%)	0.761	1.0 1.429 (0.142- 9.978)

Although JAK2^{V617F}-negative group had lower percentage in urban residence (73.68%) compared to JAK2^{V617F}-positive group (80%), the difference was insignificant (p= 0.671, OR= 1.429, 95%CI= 0.142-9.978).

Serum Level of Alkaline Phosphotase and Lactate Dehydrogenase: JAK2^{V617F}-positive group had an average level of ALP of 146.05±8.028 IU/L which is slightly above the maximum normal limit (140 IU/L), and differed significantly from JAK2^{V617F}-negative group (64.45±40.15 IU/L) (figure 2). Almost all serum samples from JAK2^{V617F}-positivegroup exceeded the higher normal limit of this enzyme (190 IU/L) and the average concentration was 204±10.85 IU/L. For JAK2^{V617F}-negativegroup, although several samples exceeded the normal limit, the average was below that limit (178.33±13.693 IU/L) and differed significantly from the first group.

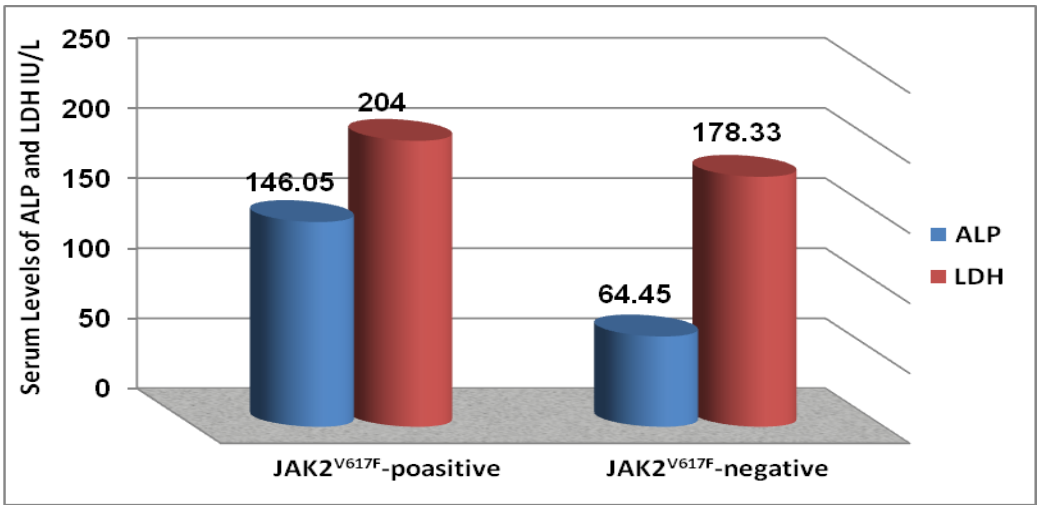

Figure 3: Serum Levels of Alkaline Phosphatase and Lactate Dehydrogenase in Chronic Myelogenous Leukemia.

DISCUSSION

The current study revealed that 11.62% of Ph+-CML patients were positive for $JAK2^{V617F}$ mutation. This result is in accordance with plethora of previous studies either as prevalence[4,7], although with different percentages, or as sporadic cases [5,6]. There was no possibility to determine which mutation has occurred first ($JAK2^{V617F}$or Bcr-Abl), however, it can be deduced that the later mutation had occurred first because the small ratio of mutant allele of $JAK2^{V617F}$ which indicates the recent occurrence and the possibility to increase over time. In fact astute insight for figure 2, could illustrate the presence of several samples beyond the recorded 5 samples which have very small ratio of mutant allele. These ratios were too small to be recorded as positive result. With the time progress, these ratios may grow larger. Many authors referred the occurrence of $JAK2^{V617F}$ after afterBcr-Abl mutation to selective treatment of Ph+CML patients with tyrosine kinase inhibitors [8]. Supporting this notion is a report which showed that, while Bcr-Abl transcripts decrease during imatinib treatment, the $JAK2^{V617F}$ mutant allele increases [9]. This assumption completely fits the idea of successive acquisition of both genomic abnormalities by one subclone of progenitor cells [10]. However, it is unfair to neglect the opposite hypothesis about the emergence of a Bcr-Abl transcript against the background of $JAK2^{V617F}$mutation. Similarly, the cytotoxic therapy used for the treatment of MPNs was also accused as a cause for such emergence [11].

Based on the previous and current result, the mutual exclusion hypothesis (which stated that either $JAK2^{V617F}$ or Bcr-Abl mutation is present in an individual at a given time) is no longer valid. It is reasonable to assume that the concomitant occurrence of the two mutation affect the patient's phenotype in a manner depends on which mutation occurs first. In case that Bcr-Abl transformation is the original abnormality, the subsequent occurrence of $JAK2^{V617F}$ mutation tends to add signs of polycythemia vera (PV) or to least extend essential thrombocythemia (ET) and idiopathic (idiopathic myelofibrosis) IMF to the original phenotype of CML. However, these chronological events can be affected by two factors; the first one is the imatinib treatment which can control the CML progression with its clinical and laboratory findings. As this drug almost has no effect on $JAK2^{V617F}$ mutation, there will preponderance of the phenotype related to the new mutation. The other factor is the ratio of burden wild allele of $JAK2^{V617F}$. Low ratio cannot induce tangible clinical or hematological changes while high ratio could induce such changes.

The reverse scenario could not be postulated for the occurrence of $JAK2^{V617F}$ before Bcr-Abl transcript. Although many diseases are associated with $JAK2^{V617F}$, the common therapy is JAK2 inhibitor (ruxolitinib)which has been used effectively in the treatment of different myeloproliferative neoplasms [12]. This drug interferes with the ATP-binding pocket and thus block JAK2 kinase activity and the resultant activation of the STAT pathways [13].Thus, there is no effect on the mutant allele and therefore there will be no favor for the increase in Bcr-Abl, and the two genetic aberrations exert their clinical and hematological effects somewhat in separated manner.

A previous work conducted by El-Hady *et al*.[14]revealed an association between $JAK2^{V617F}$ (especially homozygosity) and age. The insignificant result in the current study may be largely related to the small number of $JAK2^{V617F}$-positive cases. Anyway, the most plausible explanation for increasing of mutant allele burden of this mutation in older ages is that an age-dependent changes in DNA damage and/or DNA repair mechanisms contribute to an increased rate of mitotic recombination and acquisition of the mutation [15].

The resultant OR indicates that male has a two-fold opportunity (1/0.5) to get $JAK2^{V617F}$ than female. This result agreed with that of Godfrey *et al*[16] who reported an association between male gender and increasing granulocyte $JAK2^{V617F}$allele burden. The authors suggested a gender-related differences in the frequency of mitotic recombination as a possible cause for this association. In the current study, all $JAK2^{V617F}$ cases were heterozygous; therefore, such suggestion does not fit to explain the association. An alternative explanation may be related to nature of work which predispose for radiation and subsequent induction of different mutations including $JAK2^{V617F}$.

Obesity is a well-known risk factor for many malignancies such as breast cancer [17]. That is because excess endogenous estrogen from endogenous estrone in adipose tissue contributes in induction of estrogen receptor (ER) which increase the risk of cancer in obese women [18]. Actually bone marrows (where the $JAK2^{V617F}$ mutation usually originates) do have ER, however the majority of participant of the present study were within normal healthy weight and the logistic regression revealed no significant association.

Smoking is a leading preventable cause of many cancers such as lung cancers [19]. Nevertheless, the association of smoking with blood malignancies is less obvious. In their cohort study, Weinberg *et*

al.[20] found JAK2^{V617F} mutation in 35.5% in smokers and 14.8% in non-smokers, a result which was confirmed later by El-Hady*et al*[14] who found the mutation in 20.6% of healthy smokers and 9.5% of healthy nonsmokers. These results are extraordinary because of very high prevalence of JAK2^{V617F} mutation either among smokers or nonsmokers and needed to be confirmed by other studies. However, if the smoking has an effect, it may be attributed to notorious effect of some cigarette components on the DNA.

Urban residences expose more frequently than rural residences to different types of carcinogens like benzene which was found to increase the incidence of some JAK2^{V617F}-associated MPNs [21]. However, in an Australian case-control study, Giles *et al*[22] found that rural sector workers such as farmers were at high risk of having IMF or PV compared to control may be due to exposure to fertilizers. It seems that there is no prominent effect of rural or urban residency on the acquisition of JAK2^{V617F}because each one has its risk factors, but rural residents, generally, have lower socioeconomic status and educational level, combined with limited knowledge about mutation and cancer. Furthermore, rural residents have less accessibility to the health centers and may hesitate to consult these centers even if they had a complain unless a certain degree of injury occurs [21].

Neutrophil ALP is a minor tributary for serum ALP; however, the fraction of this tributary varies largely with the body's condition. NAP is released into circulation through in cases of damaged or dead neutrophils. The activity of NAP is substantially decreased in HSCs disorders like CML[23]. The reason beyond this reduction is believed to be a consequence of NAP messenger RNA deficiency in the malignant cells [24]. Nevertheless, the present result indicates an average of serum ALP activity in JAK2^{V617F}-negative CML patients within normal limits which may be due to the effect of imatinib which was used by all patients. This effect is formerly confirmed by Al-Shami [25] who examined the side effects of imatinib in Iraqis CML patients and found an elevation in APL in some patients.

On the other hand, most JAK2^{V617F}-positive had serum ALP activity higher than the normal limit. This result is in accordance with [26, 27]who recorded an elevation of ALP activity associate with JAK2^{V617F} mutation. Furthermore, Asignificant relationship between percentage of JAK2 mutant alleles and ALP expression was found; high ALP scores can predict JAK2 mutation [26]. It is thought that induced production of neutrophils by G-CSF is the main cause of increased NAP and consequently serum ALP activity [28].

LDH plays a key role in regulating glycolysis by catalyzing the final step of anaerobic glycolysis, therefore, its upregulation facilitates the efficiency of anaerobic glycolysis in tumor cells and reduce their dependency on oxygen [29]. Several published works have recorded mild elevation in serum level of this enzyme in CML patients [30,5]. The higher level of LDH in JAK2^{V617F}-positive compared to the negative group can be explained by a somewhat complex mechanism which involves the constitutional activation of STAT5 by a mutated JAK2 gene. One target gene of activated STAT5 is hypoxia inducing factor alpha 2 (HIFα2) gene. This gene encodes for HIFα2 which is considered as transcriptional factor [31]. HIFα2 has been shown to control directly on the transcription of LDHA [32].

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