A physiological and molecular study of leukemia patients in Kirkuk city
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Abstract:
Aim of the study: Determination of liver enzyme [AST(GOT),ALT(GPT),ALK] and evaluate the performance of different JAK2 V617F mutation PCR:PCR-ARMS,PCR-RFLP,Real Time-PCR .The patients and methods of work collected samples from the Center for Hematology and Oncology in Kirkuk City, the study included (88) patients whose ages ranged between (2-79) and also included (40) of natural persons as control cases whose ages ranged between (7-63). In molecular tests, (75) a patient sample of the total patient samples was used. The Determination of liver enzyme (AST,ALT,ALK) was performed using several equipment provided by the company (randoxs and Biomerieux) for the studied enzyme, Real-time examination was done using a kit equipped by (Bioneer-Korea) company. Results: the results showed the increase in liver enzymes in patients (male and female) compared to (male and female) control at the level of significance. As for the results of molecular tests, the results showed that using the assay (RFLP) that 22 samples gave genotype (GG) and 53 samples gave genotype (GT), as for the assay (ARMS), all samples gave the genotype (GT) Conclusions :High liver enzymes in leukemia patients.

Key words : Leukemia,liver enzyme, JAK2 V617F mutation,real time-PCR


Introduction:
Leukemia is the cancer of the bone marrow and the white blood cells, (WBCs). Although leukemia is considered as a dangerous type of cancer, the recent advances and development in the diagnostic tools and treatment options have resulted in a cure rate of almost 50%(1). Generally speaking, there are two types of leukemia; namely acute leukemia(Acute lymphoblastic leukemia ALL, Acute Myeloid Leukemia AML) and chronic leukemia (Chronic lymphocytic leukemia CLL,Chronic Myeloid Leukemia CML). Acute leukemia is clinically and biologically different from chronic leukemia .Acute leukemia is characterized by its rapid and aggressive proliferation of immature cells, namely, the blast cells. On the other hand, chronic leukemia progresses slowly over the course of many years(2).

JAK2 gene
JAK2 :Janus kinase 2 a cytoplasmic tyrosine kinase is situated on chromosome 9p24 and contains 25 exons and its protein 1132 amino acids. JAK2 is the 1 of 4 mammalian JAKs (JAK.1, JAK.2, JAK.3, and TYK.2)(3) show an essential part in signaling cytokine in hematopoietic cells (4). activation of JAK is mediated via ligand-induced ingathering of cytokine receptor –related JAKs and their subsequent autophosphorylation at specified tyrosine remains. These phosphorylated tyrosine residues avail as docking sites of SH2 domain – include proteins of the family of STAT (5). On binding of cytokines like interleukin-3, erythropoietin, and granulocyte-macrophage colony stimulating factors (GM-CSF) and granulocyte colony stimulating factors (G-CSF) the activated JAK2 tyrosine residues phosphorylate the cytoplasmic portions of the cytokine receptors –induced phosphorylation of these cytokine receptors is required for their interaction with the JAK2 molecules. In this way, JAK2 serves as a signaling molecule downstream of the cytokine receptors.
parameter, STAT monomers are phosphorylated via JAK.2 and dimerize and translocate to the nucleus, wherever they modulate gene transcription. (6)

JAK stransduce signals of their cognate kind I and kind II non kinase cytokine receptors. Discer ning relationship of a JAK family member for especific cytokines or grow th parameter may explainsome of the variances in therapeutic and side-affect pro filesamong drugs that primarily target JAK.1, JAK.2, JAK.3 or multiple JAKs (7).

**JAK2V617F**

Oncogenic JAK.1, JAK.2 and JAK.3 mutations has linked for both myeloid neoplasms and lymphoid. Of the special relationship with MPN, JAK.2V617F was discovered in2004 and initial reports show in early 2005.(8). JAK.2V617F is through far the utmost prevalent mutation in BCR-ABL1-negative MPN (occurs in B95% of patients for PV, in B55% with ET and in B65% with PMF), but it is too seen in some patients of myelodysplastic syndrome (MDS)/MPN (like, thrombocytosis and refractory anemia with ring sideroblasts ) infrequently, in primary (AML), MDS or CML. Though, this wouldnt undermine its broad especificity to patients for myeloid neoplasms (counting those with occult disease and splanchnic vein thrombosis)( 9, 10) The mutation effects the noncatalytic ‘pseudo-kinase’ range and is think to derail its kinase-regulatory pep. JAK.2V617F-mediated transformation is think to request coexpression of kind I cytokine receptor and leads to STAT5/3 activation((11,12). thus Some patients for MPN may transport JAK.2 mutations multiple, some times befalling in the same exon and in cis order,. like events may has functional relation they may alter particular signaling. JAK.2V617F induces PV-like phenotype in mouse transplanta tion models, and this observation have additional inveterate via a recent report of an inducible JAK.2V617F knock-in mouse model, that both homozygous mutation and heterozygous expressions induced PV-like disease, of the latter causing a most phenotype aggresse for myelofebrosis (13,14).

**Material and Methods**

**Collection of Sample:**

Serum was collected from (128) a representative sample of 88 sample of leukemia (49 males and 39 females, age ranged from 2-79 years) and 40(19 Male and 21 female ,age ranged from 7-63years) healthy samples from the Center for Hematology and Oncology from 8-2017 to 3-2018. The disease was clinically diagnosed by the consultant medical staff at the centre.

The study Sample were divided into:- First Group , healthy control.. Second Group , who have leukemia patients to divide two group :(Acute+Chronic),Acute leukemia patients : (ALL+AML): A:ALL(21 Male+10 Female),Chronic leukemia patient(PLL+CML): A:PLL(8 male+9 female), B:CML(13 male+11 female). In molecular tests, (75) a patient sample of the total patient samples was used(ALL 16 male+9 female,AML5 male+6 female,PLL10 male+8 female,CML10 male+11 female).

**Determination of liver enzyme(AST,ALT,ALK):according to standard procedure**
DNA extraction method:
Withdraw (400) from the blood and placed in a 2 ml tube and added to it (600) from the erythrocyte solution, placed the tubes on the vibrator for five minutes for the purpose of mixing and analysis of erythrocyte. Centrifuge the tubes at (14000) for five minutes. Was clear of the odor and retain the precipitate and then add (300) from the solution of analysis of erythrocytes and mix the precipitate using a fine pipette and then carburetor to break up the precipitate. Centrifuge the tubes at speed (14000) for five minutes. Was cleared of the stew and then add (300) normal saline and mix the precipitate using a fine pipette and then carburetor to break up the precipitate. Centrifuge the tubes at a speed of (1400) for five minutes. Repeat the previous steps until we obtain a pure white precipitate representing white blood cells free of hemoglobin. Add (400) of the solution of the analysis of the components of the cell previously placed in the water bath and prick the sediment using a pipette and then vortex to break up the precipitate. Pipes were placed in a water bath (55) for an hour. Then let the tubes to cool at room temperature. Add (400) chilled chloroform and (50) sodium chloride solution and left on the vibrator for a quarter of an hour and then centrifuge the tubes at a speed (14000) for ten minutes at four degrees. Transfer (300) from the upper water layer to a new pipe. Add twice the volume transferred (600) of the cooled absolute ethanol and (50) of the ammonium acetate solution and left for a quarter of an hour at zero temperature after which the tubes are quietly upside down for DNA deposition. Centrifuge the tubes at a speed (14000) for five minutes at 4 ° C and then get rid of the air and keep the sediment. Washing the precipitate with (70%) ethylc hol, centrifuging and centrifuging the tubes at a speed of (14000) for five minutes at 4 ° C. Then the precipitate is dried by leaving the tubes for an hour in the incubator at 37 degrees or by leaving them at room temperature until the ethanol is dry. Add (150) of solution (TE buffer) to the extracted DNA for the purpose of dissolution and then developed. Pipes in the water bath at 65 for a quarter of an hour or for two hours incubator at the degree of 37.(15)

JAK2 V617F mutation by restriction fragment length polymorphism (RFLP):
There was a multiplicity of the forms of the gene by using the indicator (PCR-RFLP), as the piece of the gene msssmultiplied to be studied using specialized prefixes and through interactions (PCR), and then it reveals the presence of the mutation or its absence through the use of cutting enzymes that recognize specific sites within the double segment and cut it. The reaction was carried out using kit (PCR-Premix) equiped from (Bioneer) company and with a final volume (20µl) and using the specialized primar:primers sense (5′ TGC TGA AAG TAG GAG AAA GTG CAT 3′) Antisense primer (5′ TCC TAC AGT GTT TTC AGT TTC AA 3′).to amplify a piece of DNA, the reaction components, Genomic DNA 100 ng, primers sense1 µM, antisense primer1 µM, dNTPs200 µM MgCl 21.5 Mm.the cycling conditions was as follows: 1 cycle of 95 °C of 2 mint.; 35 cycles of 94 °C , 30 s, 56 °C of 1 mint., 72 °C of 1 mint., and a finishing extension at 72 °C of 5 mint.. After that, the reaction data is interrupted via addition 5 units of the restriction enzyme(BsaXI) to each sample and incubation at a degree (37) for a period of three hours. The product resulting from cleavage with the enzyme was loaded on a 3% agarose gel,ethidium promid staining and subjected to electrophoresis.
PCR product showed three types of genotypes. Normal Homozygous (GG) is a bundle of size Molecular 180 bp, Heterozygous (GT) is a bundle of size (180bp,360bp) . Mutant Homozygous (TT) is a bundle of size (360) bp.(11).

**V617F genotyping by amplification refractory mutation system (ARMS):** Optimization of pcr reaction was accomplished, thus the following program was adopted. The reaction was carried out using kit (PCR-Premix) equiped from (Bioneer) company and with a final volume (20µl) and using the specialized primar forward outer (FO), 5-TCCTCAGAACGTTGATGGCAG-3. reverse oute (RO), 5-ATTGCTTTCCTTTTCACAAGAT-3. forward wild-type–specific (Fwt), 5-GCATTTGTTTTAAATTATGGAGTATTaTG-3. reverse-mutant–specific (Rmt), 5-GTTTTAICTTACTCTGTCMCAACAAlAA-3.(16).The cycling conditions follows: 1 cycle of 95 °C of 2 mint.s; 35 cycles of 94 °C , 30 s, 56 °C of one min., 72 °C of 1 mint., and a finishing extension at 72 °C of 5 mint. PCR products of tetra amplification refractory system polymerase chain reaction verified on 3% agarose gel contained ethtidium promid stain at 5vol/cm.for one and half hours and then photograph were taken. PCR product showed three types of genotypes. Normal Homozygous (GG) is a bundle of size Molecular 230 bp, Heterozygous (GT) is a bundle of size (230bp,277bp) . Mutant Homozygous (TT) is a bundle of size (277) bp.(11).

**Quantitative Real Time:** Here, we utilized the previously published forward primer: 5′ AGC AAG CTT TCT CAC AAG CA 3, reverse primer: 5′ CTG ACA CCT AGC TGT GAT CC T G 3′. Primer utilized with housekeeping gene (Beta globin PCO3):

forward primer: 5’ ACA CAA CTG TGT TCA TCA GC-3 reverse primer: 5’ CAA CTT CAT CCA CGT TCA CC-3’. Protocol:

Add following pcr reagent in to green star qpcr premix tube (pre reaction)

<table>
<thead>
<tr>
<th></th>
<th>20 µl rxn</th>
<th>50 µl rxn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pcr f-primer</td>
<td>1-2 µl</td>
<td>1-2 µl</td>
</tr>
<tr>
<td>Pcr r-primer</td>
<td>1-2 µl</td>
<td>1-2 µl</td>
</tr>
<tr>
<td>Template</td>
<td>5- 10 µl</td>
<td>5-10 µl</td>
</tr>
<tr>
<td>D.W.</td>
<td>Adjust to 20 µl</td>
<td>Adjust to 50 µl</td>
</tr>
</tbody>
</table>

We quantified gene expression levels and fold change by measuring the threshold cycle (Ct) that defined as the PCR cycle at thus the signal fluorescent of the reporter dye crosses an arbitrarily placed threshold. We used classic comparative Ct way to determine the expression level of the gene of interest comparative to a calibrator or sample reference utilizing the Ct data below (17).

\[
\Delta CT \text{ sample} = \text{Ct sample} - \text{Ct control endogenous} \\
\Delta CT \text{ calibrator} = \text{Ct healthy control} - \text{Ct control endogenous}
\]

The Ct data were normalized by using endogenous control genes(ABL and miR-16 genes). Normalization parameter is the arithmetic mean or geometric mean of Ct values of the designated genes control. The normalized \(\Delta CT\) data are utilized to evaluate the comparative gene

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expression fold change utilizing a designated calibrator (healthy or sample reference): \( \Delta \Delta CT = \Delta CT_{sample} - \Delta CT_{calibrator} \), Fold Change = \( 2^{-\Delta \Delta C} \).

**Results and Discussion**

- **Result of liver enzyme**: In the present study, Table 1 and 2 show liver enzymes activities in leukemic patients and controls subjects of both gender.

**Table 1- Liver enzymes in male patients and controls.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>ALL</th>
<th>AML</th>
<th>CML</th>
<th>CLL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Got</td>
<td>8.27±2.07</td>
<td>15.90±3.99</td>
<td>15.42±3.87</td>
<td>12.46±3.10</td>
<td>16.00±4.2</td>
</tr>
<tr>
<td>GPT</td>
<td>8.9±2</td>
<td>12.88±3.20</td>
<td>13.42±3.32</td>
<td>14.15±3.55</td>
<td>12.75±3.16</td>
</tr>
<tr>
<td>ALK</td>
<td>7.43±1.71</td>
<td>16.44±4.90</td>
<td>18.17±4.50</td>
<td>15.66±3.93</td>
<td>6.68±1.65</td>
</tr>
</tbody>
</table>

There are significant elevations in the activities of ALK, GOT and GPT of leukemic patients, as compare with healthy control subjects, (p≤0.01)

**Figure-1- Liver enzymes in male patients and controls**

**Table 2- Liver enzymes in female patients and controls**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>AML</th>
<th>ALL</th>
<th>CLL</th>
<th>CML</th>
</tr>
</thead>
<tbody>
<tr>
<td>Got</td>
<td>7.75±1.94</td>
<td>20.3±5.08</td>
<td>38.5±9.6</td>
<td>10.7±2.8</td>
<td>10.5±2.7</td>
</tr>
<tr>
<td>GPT</td>
<td>7.9±1.98</td>
<td>19.1±4.9</td>
<td>23.8±7.4</td>
<td>12.6±3.15</td>
<td>11.0±2.8</td>
</tr>
<tr>
<td>ALK</td>
<td>7.1±1.6</td>
<td>12.7±3.2</td>
<td>19.3±4.6</td>
<td>14.7±3.3</td>
<td>14.0±3.3</td>
</tr>
</tbody>
</table>

There are significant elevations in the activities of ALK, GOT and GPT of leukemic patients, as compare with healthy control subjects, (p≤0.01)

**Figure-2- Liver enzymes in female patients and controls**

The levels of AST, ALT(AL.T) is a extra specific marker than AS.T of liver injury then it is utmost find in liver tissue, localized to the hepatocyte cytosol. though AL.T iso enzymes are express
ed in many tissues, raised serum ALT activity is deliberated the “gold standard” clinical marker of liver injury in humans (18) and ALK. Increased enzyme in patients might occur due to different parameter counting leukemic cells infiltration of the liver (infiltration disorder as a result of a defect in the membranes of mitochondria and cytoplasm (19), therapy-related side effects like nephrotoxic drugs tumor lysis syndrome, and septicaemia. (18). The elevation in ALK activity occurs because of the accelerated denovo synthesis of the enzyme or because the rise of tumor necrosis factor-α and its direct effect on the expression of bone phosphatase in vascular smooth muscle cells as well as how ALP effects the inflammatory responses and might play a direct part in preventing organ damage (20).

**Polymorphism of JAK2 gene**

**RFLP analysis of JAK2 V617F mutation**: the exploration results of the *BsaXI* enzyme PCR product showed three types of genotypes. Normal Homozygous (GG) is a bundle of size molecular 180 bp, Heterozygous (GT) is a bundle of size (180bp, 360bp) . Mutant Homozygous (TT) is a bundle of size (360) bp. The replacement mutation of the nitrogen base G, which converts it to base T, at the site 14 exon. To harvest JAK2 has created a cutting site for the cutter breeze *BsaXI*.

![RFLP electrophoresis](image)

**Figure 3:** The electrophoresis of (PCR-RFLP) a gene (JAK2) on the acarose gel (3%).

According to the method (RFLP) 53 samples gave the genotype (GT) and 22 samples gave the genotype (GG).

**ARMS analysis of JAK2 V617F mutation**: the exploration results of the PCR product showed three types of genotypes. Normal Homozygous (GG) is a bundle of size molecular 230 bp, Heterozygous (GT) is a bundle of size (230bp, 277bp) . Mutant Homozygous (TT) is a bundle of size (277) bp. The replacement mutation of the nitrogen base G, which converts it to base T, at the site 12 exon.
Real Time -PCR

Gene expression was discovered using a molecular technique (Qrt-pcr) with the use of a custom initiator as described in Chapter Two. The amplification accuracy of a gene product was observed by the cycle threshold value (Ct) for interactions.

The results found of the qRt-PCR experiments according to the values of Ct calculated from the cycles were proportional to the number number of copies of the target to be amplified (logarithmic scale) which is inversely proportional to the starting template quantity which means that the rise in the value of Ct indicates Low levels of gene expression or genes have been amplified, while a low Ct value indicates a high level of gene expression or a high number of copies of genes that have been amplified. Amplified graphics appeared when the fluorescent signal (fluorescence) was drawn from the sample.
with the cycle number; however amplified drawings may include product duplication and accumulation during the qPCR reaction period.

Real time of patients (n=70): 10.23±2.57

Some samples did not give results, which may be due to an experimental error.

**Genotypes and liver enzymes by (RFLP assay):**

<table>
<thead>
<tr>
<th>Variables</th>
<th>GG(n=22)</th>
<th>GT(n=53)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOT</td>
<td>22.50±5.00</td>
<td>14.00±3.3</td>
</tr>
<tr>
<td>GPT</td>
<td>16.20±4.00</td>
<td>13.42±3.34</td>
</tr>
<tr>
<td>ALK</td>
<td>17.73±4.41</td>
<td>15.07±3.73</td>
</tr>
</tbody>
</table>

**Correlation of the gene with The relationship of the gene with liver enzymes**

The Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway is one of the main inflammatory pathways marking downstream of cytokines. Activated the liver, JAK.2 is via various cytokines and growth agent inclusive IFN, IL-4, IL-6, IL-12, IL-13, growth hormone (GH), and leptin (21). Disruption of hepatic leptin marking confirmed intrahepatic lipid cumulation but protect ed of diet- and age-induced glucose intolerance (22).

According to (23) function Hepatic JAK.2 in the organization of carbohydrate and lipid inflammation and metabolism. Micelacking JAK.2 in hepatocytes presented with profound spontaneous hepatic steatosis connected of tenderized insulin stimulated phosphorylation of Akt in the liver; yet, this flooding lipid cumulation did not progress to steatohepatitis or all-body resistance insulin on a HFD. moreover both raised signaling GH and signaling of the dampened hepatic insulin may mediate compensative proliferation cell in restraint to HFD feeding, performance L-JAK.2 KO mice resistant to expansion of diet-induced glucose intolerance (24).

**Conclusions**

The results showed the increase in liver enzymes in patients (male and female) compared to (male and female) control at the level of significance. As for the results of molecular tests, the results showed that using the assay (RFLP) that 22 samples gave genotype (GG) and 53 samples gave genotype (GT), as for the assay (ARMS), all samples gave the genotype (GT).

**References:**


