Detection of mutation in Exon 2-3 in *Perforin* gene and Exon4 in *Fas* gene in sample leukemia Iraqi patients

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ABSTRACT

This work aimed to study the possible mutations in *Perforin* gene (*PRF1*) exon 2-3 and *Fas* gene exon 4 in Acute Lymphocytic Leukemia (ALL) and Chronic Lymphocytic Leukemia (CLL) patients from Iraq. In an attempt to detect any mutation within *PRF1* gene and *Fas* genes, a sequencing analysis for these genes were made. The results were alignment with sequences present in the Gene Bank seeking for homology and differences. A DNA sequence for *Homo sapiens PRF1* gene was found compatible with genes of ALL, CLL patients and healthy controls, 100% compatibility was found in the flank DNA sense and antisense sequences from healthy. However, 99% compatibility was detected for the genes isolated from ALL patients with an insertion of C697 and A698 G in the flank DNA sense strand and insertion of G697 and T698C in flank DNA antisense strand of the gene. Morever, 99% compatibility was detected for the genes isolated from CLL patients with two transition mutations in the flank DNA sense strand of C957T and C1035T and one transition mutation in the flank DNA antisense strand of G957A. However, no mutations were detected in *Fas* gene isolated from ALL, CLL, and healthy controls.

INTRODUCTION

In humans, perforin deficiency leads to a potentially fatal disorder in infancy, familial hemophagocytic lymphohistiocytosis type 2 (FHLH2) (1). Patients with mutations in the perforin gene (*PRF1*) have absent or low perforin levels in NK cells and diminished lymphocyte cytotoxicity (2). Missense mutations in *PRF1* have also been described in an adult with chronic active epstein barr virus (EBV) infection (3) and in children with bone marrow malignancies (4). The phenotypic expression of *PRF1* mutations is variable, and that the spectrum of perforin-related disease may include fatal immune dysregulation in early childhood; nonfatal, inflammatory reactions at any age and impaired tumour surveillance in children and adults. In other studies of FHLH2, over 50 mutations of the perforin gene (1 & 5) have been identified, most of the perforin mutations in patients with FHLH2 do not lead to severe protein truncation but consist of amino acid substitutions and detection of mutant perforin by Western bloting of perforin lysates from individuals (6). Missense mutations in perforin, a critical effector of lymphocyte cytotoxicity, lead to a spectrum of diseases, from FHLH2 to an increased risk of tumorigenesis (1). Most missense *PRF1* mutations in FHLH2 patients result in loss of function of perforin, most commonly due to unfolding and faulty trafficking of the protein (7 & 8), the mutation identified in perforin result in loss of a functional mRNA and complete loss of perforin protein or non functional protein (9 & 10). The present study aimed to investigate correlation between mutation in *PRF1* and *Fas* gen and increasing leukemia in Iraqi population.

Materials and methods

Collection of samples

Five ml of blood was collected by vein puncture from 39 cases (21 ALL and 18 CLL) who were admitted to the National Center of Haematology/ Al Mustanisyria University. The disease was clinically diagnosed by the consultant medical staff at the centre. In addition, 5 apparently healthy controls (blood donors) were also included.

Isolation of Lymphocyte

Preparation of solutions and media were done according to the methods described by{11}{12} unless mentioned. The lymphocytes were isolated from the heparinized whole blood using the method described by {13} as follows: three ml of blood was centrifuged at 1000rpm for 15min. The plasma was collected for perforin estimation, buffy coat was collected in a 10 ml centrifuge tubes and diluted with 5ml RPMI 1640 (cell suspension), five ml of the diluted cell suspension was layered on 3ml of ficoll-isopaque separation fluid, the tubes were centrifuged at 2000rpm for 30min in a cooled centrifuge at 4ºC. After centrifugation, the mononuclear cells were visible

as cloudy band between the RPMI1640 and lymphoprep layers. The band was collected in a 10ml test tube and the cells were suspended in 5 ml RPMI 1640. The tube was centrifuged at 2000rpm for 5min (first wash), the supernatant was discarded and the cells were resuspended in 5 ml RPMI 1640 (repeated twice). The suspension was centrifuged at 1000rpm for 10min, the supernatant was discarded. The precipitated cells were resuspended in 1ml RPMI solution and used in the planned experiments. Counting the cells were performed before experiment according to {13}, the numbers of lymphocytes were counted by light microscope and the cells concentration was adjusted to 1X106 cell/ml. The isolated cells were grown in a flask containing 10ml RPMI 1640 medium supplemented with BSA and incubated at 37ºC for 48h.

Isolation of Genomic DNA

Genomic DNA was isolated from culture cells under aseptic condition according to the protocol described by promega company for wizard genomic DNA purification kit (Cat #: A1120). Cells grown as liquid culture were harvested by centrifugation at 13000–16000 rpm for 10sec, the cell pellets were resuspended in PBS and vortex mixed. Nuclei lysis solution 600µl was added to cells grown as liquid culture and mixed by pipetting. RNase solution 3µl was added to the cell nuclei lysate and mixed, then incubated for 15–30min at 37°C and then cooled to room temperature at 25°C. Protein precipitation solution 200µl was added, vortex and chilled on ice for 5min, then centrifuged at 13000–16000 rpm for 4 min, supernatant was transferred to a tube containing 600µl isopropanol at room temperature, mixed by inversion and centrifuged at 13000–16000 rpm for 1min. The supernatant was removed and the pellet was resuspended in 600µl of 70% ethanol and mixed well then centrifuged at 16000 rpm for 1min. The ethanol a spirited and pellet was air-dried, the 100µl of DNA rehydration solution was added to dissolve the pellet.

Detection of *Perforin* Gene and *Fas* Gene by Using PCR

A 572 bp fragment containing exon 2-3 of PRF1 was amplified using a forward primer (5′-ACGGCAGCATCTCTGCCGAA-3′) and a reverse primer (5′GGGGTTGTTATTGTCCCACA-3′) and 272bp fragment containing exon 4 of Fas was amplified using a forward primer (5′-AATCCATGCAGCTCCTGCC -3′) and a reverse primer (5′- AGTCAGTGTTACTTCCCTAGGA 3′) (Primers set supplied by first base Company, Malaysia). The PCR amplification was performed in a total volume of 25µl containing 2µl DNA (conc. 100 ng/µl), 12.5 µl Go Taq green master mix 2X (green maschuiter mix is a premixed ready to use solution containing Taq DNA polymerase, dNTPs, MgCl2 and reaction buffers at optimal concentrations for efficient amplification of DNA template by PCR supplied by promega (Promega corporation, USA), 1µl of each primer (10 pmol/µL) and up to 25µl with nucleases free water. The thermal cycling was as follows of PRF1 exon 2-3 gene: Denaturation at 95 °C for 4 min, followed by 35 cycles of 94 °C for 40sec, 56°C for 30sec, and 72 °C for 45sec, with final incubation at 72 °C for 5min [14] using a thermal Cycler. The thermal cycling was as follows of Fas exon 4 gene: Denaturation at 96 °C for 3 min, followed by 35 cycles of 94 °C for 35sec, 63°C for 35sec, and 72 °C for 35sec, with final incubation at 72 °C for 5min [9] using a thermal Cycler. The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by exposure to ultraviolet light (302nm) after ethidium bromide staining.

Sequencing and Sequence Alignment

Sequencing of exon 2-3 of perforin gene and exon 4 of Fas gene were done by First base company/Malaysia for sequencing of products through used individual sense and antisense primer were used in each sequencing reactions. Homology searches were conducted between the sequence of standard gene BLAST program which is available at the national center biotechnology information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) and using BioEdit program.

Results and discussion

Amplification of Exon 2-3 *Perforin* Gene

The genomic DNA from 39 patient were extracted using wizard genomic DNA promega, PRF1 gene from genomic DNA were amplified by using specific PCR primers for exon 2-3, results shown in figure (1) indicated that a yield of single band of the desired product with a molecular weight about 572 bp for exon 2-3 gene was obtained.

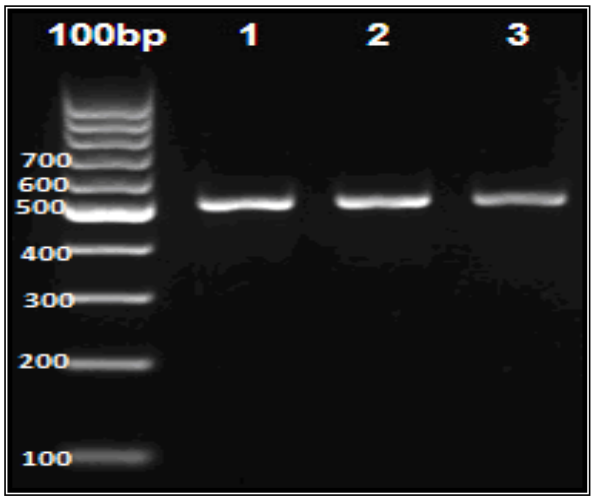


Figure (1) : Agarose gel electrophoresis for amplified *PRF1* gene (Exon 2-3) of lymphocyte belonging to healthy, ALL, and CLL patients was done. Bands were fractionated by electrophoresis on a 1.5 % agarose gel (2 h., 5V/cm, 1X Tris-acetic buffer) and visualized under U.V. light after staining with ethidium bromide staining. Lane M:100bp ladder. Lane:1.(Healthy), Lane:2.(ALL), Lane:3.(CLL).

Sequencing of coding regions of the amplified product (Exon 2-3) for these samples were done seeking for detection of any mutation within these sequence related to cancer development. Alignment of *PRF1* gene of all groups (Healthy, ALL, and CLL) with data published for known sequence seeking for enough homology. A homology with *PRF1* gene of *Homo sapiens* from the Gene Bank was done using the BioEdit software. 100% compatibility of that gene was found with *PRF1* gene (flank DNA sense and antisense of the gene) from healthy with standard *PRF1* of Gene Bank results as shown in figure (2).

A: Sense of the partial *PRF1* gene.

Score = 750 bits (406), Expect = 0.0 , Identities = 406/406 (100%), Gaps = 0/406 (0%), Strand=Plus/Plus

B: Antisense of the partial PRF1 gene

Score = 689 bits (373), Expect = 0.0 ,Identities = 373/373 (100%), Gaps = 0/373 (0%)

Strand=Plus/Minus

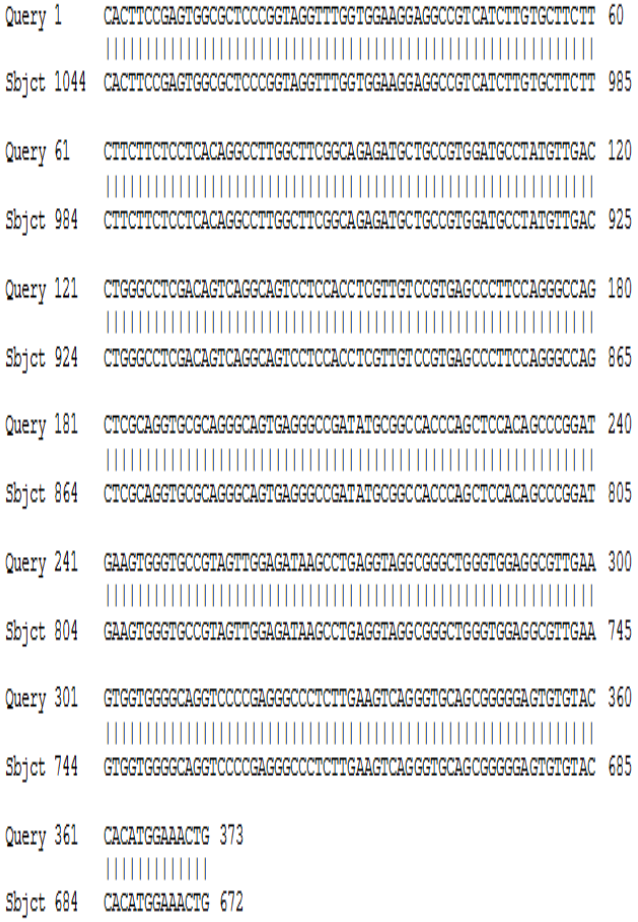


Figure (2): Sequencing of sense and antisense flanking the *PRF1* gene for healthy as compared with standard *PRF1* obtained from Gene Bank (A: Sense of the gene; B: Antisense of the gene).

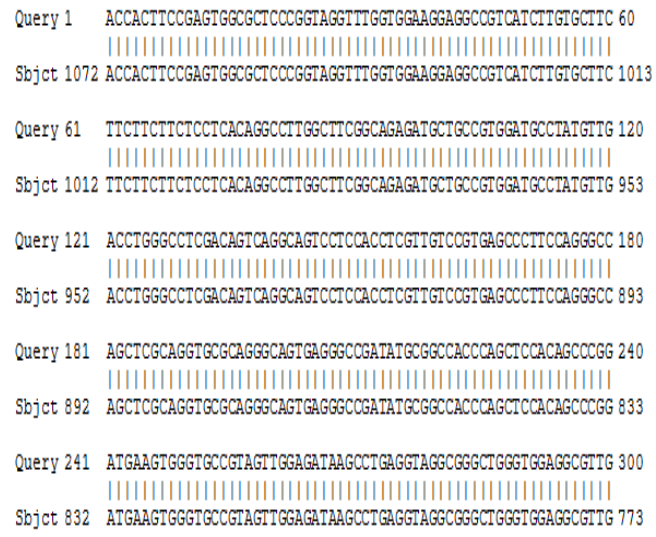
The *PRF1* gene from ALL patients showed 99% compatibility with standard *PRF1* of Gene Bank, and there was insertion of C 697 and A 698 G in the flank DNA sense and insertion of G 697 and T698 C in flank DNA antisense strand leading to change all codons (framshift mutation). This can change amino acid and the effect could impair the function of perforin and cause problems in metabolic activity and effect function as shown in figure (3).

A: Sense of the partial *PRF1* gene.

score = 750 bits (406), Expect = 0.0,Identities = 411/413 (99%), Gaps = 1/413 (0%),Strand=Plus/Plus

B: Antisense of the partial *PRF1* gene.

Score = 693 bits (375), Expect = 0.0, Identities = 380/382 (99%), Gaps = 1/382 (0%), Strand=Plus/Minus.



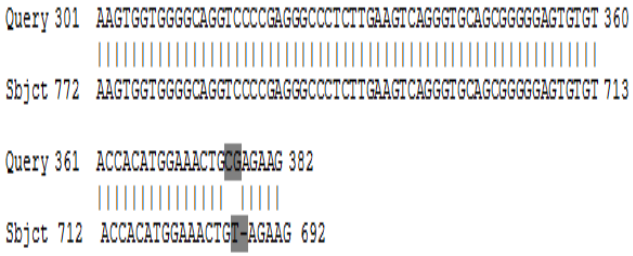
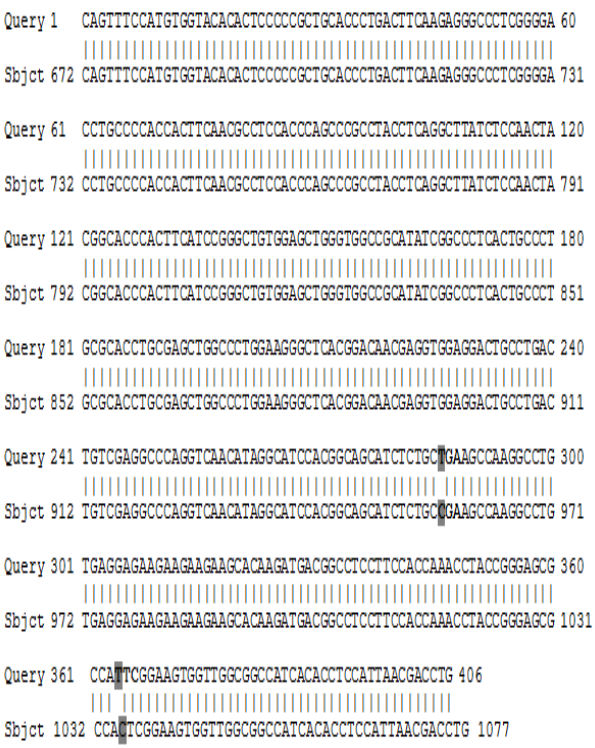


Figure (3): Sequencing of sense and antisense flanking the PRF1 gene for ALL patient as compared with standard PRF1 obtained from Gene Bank (A: Sense of the gene; B: Antisense of the gene).

The *PRF1* gene from CLL patients showed 99% compatibility with standard *PRF1* of Gene Bank. There were two transition mutations in the flank DNA sense strand C957 T and C1035 T while there are one transition mutation in the flank DNA antisense strand G 957 A as shown in figure (4); table (1).

A: Sense of the partial *PRF1* gene.

Score = 739 bits (400), Expect = 0.0 ,Identities = 404/406 (99%), Gaps = 0/406 (0%) , Strand=Plus/Plus



B: Antisense of the partial *PRF1* gene.

Score = 684 bits (370), Expect = 0.0 ,Identities = 372/373 (99%), Gaps = 0/373 (0%), Strand=Plus/Minus

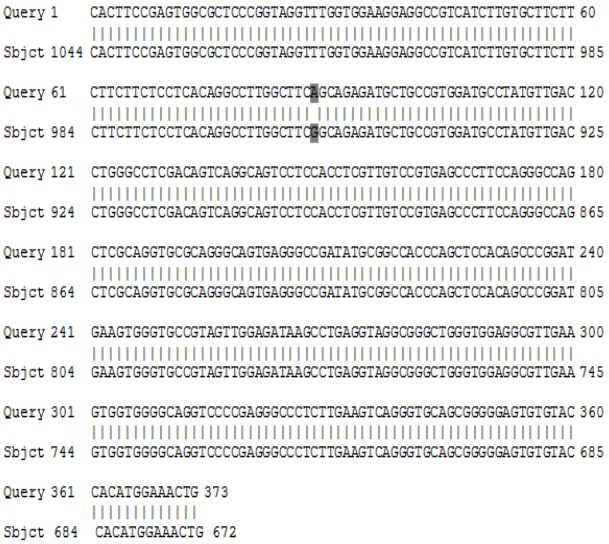


Figure (4): Sequencing of sense and antisense flanking the PRF1 gene for CLL patient as compared with standard PRF1 obtained from Gene Bank (A: Sense of the gene; B: Antisense of the gene).

Table (1): Types of mutations detected in partial PRF1 gene of CLL patients.

No.	location of gene bank	Nucleotide change	No. of codon/ location	Amino acid change	Predicted effect	Type of mutation
1	C 957T	CGA>TGA	96/Sense	Arginine>Opal	Nonsense	Transition
2	C1035T	CTC>TTC	122/Sense	Leucine >Phenylalanine	Missense	Transition
3	G 957A	GGC>AGC	30/Antisense	Glysine>Serine	Missense	Transition

Human perforin gene mutations were detected previously by several investigators. For instance, nine types of nonsense mutations and other four types of missense mutations were characterized in three cases from Japanese patients suffering from familial hemophagocytic lymphohistiocytosis FHLH2 (15). Also mutations in 12 base pair (codon 284-287) which are responsible for the change in four amino acids of the complex domain of a membrane protein were detected from Omani boy diagnosed at 44 days after birth suffering from FHLH2 disease (16) . Later, many mutations were detected in the *PRF1* gene from eleven patients (six males and five females) during DNA sequencing of exon 2 and exon 3, of these mutations (17), seven different changes were identified in the coding region of the perforin gene, five of them (265C>A, 518C>T, 363C>T, 674 G>C and deletion 12 bp) are novel along with other (50 deletion T and 1122G>A). During this review, they recognized 40 mutations within coding region of *PRF1* gene in different ethics groups, but seven different mutations in the *PRF1* gene in Omanis determined clinically to have FHLH2 with a family history. (5) referred to perforin mutation identified in 7 of the 34 families FHLH2 of Turkey investigated, six children were homozygous for the mutations and one patient was a compound heterozygote, four novel mutation were detected (one nonsense, two missense, and one deletion of one amino acid). On the other hand, referred to a mutation in exon 2 (del207C) and exon 3(del 1090-91CT) were detected in FHLH2 patient from Japan lead

to lower expression of perforin from lymphocyte of the patient (18). Also, one mutation in *A91V* (C to T transition at position 272) in perforin gene was detected during a study on 30 cases of childhood acute lymphocytic leukemia (ALL) and *A91V* frequency was significantly increased in childhood ALL but *A91V* polymorphism was not associated with increased risk (19). Moreover, three heterozygous mutations were detected in a coding region of perforin gene in three patients of hemophagocytic lymphohistocytosis (14). While, 21 missense mutations in perforin gene of hemophagocytic lymphohistocytosis patients lead to absent or low levels of perforin in NK cells (20).

During a study on 60 cases familial hemophagocytic lymphohistiocytosis (FHLH2) 22 missense mutations were detected (*P39H, G45E, V50M, D70Y, C73R, W95R, G149S, F157V, V183G, G220S, T221I, H222R, H222Q, I223D, R232C, R232H, E261K, C279Y, R299C, D313V, R361W* and *Q481P*) in *perforin* gene that lead to reduce or absence of perforin activity (8). Through their diagnosis of 9 Turkish patients suffering from FHLH2, a research group (1) identified five nonsense mutations *W374X* and four different missense mutations namely *G149S,V50M, A91V* and *A523D*. Other mutations were also detected by others investigator that reduced the functional activity and perforin expression such as *A91V* mutation in NK and CD8+ cells (21) and frame shift mutation in perforin gene leading to stop codon which cause loss of perforin functional activity (10).

Amplification and Sequencing of Partial *Fas* Genes

Fas gene from genomic DNA were amplified by using specific PCR primers for exon 1, results shown in figure (5) indicated that a yield of single band of the desired product with a molecular weight about 272 bp for exon 1 gene was obtained.

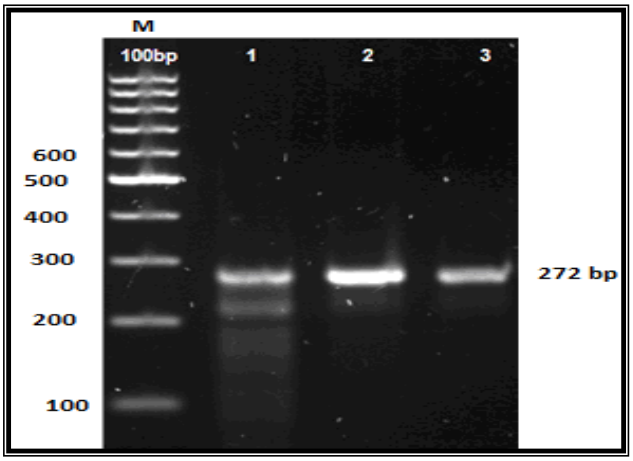


Figure (5) : Agarose gel electrophoresis for amplified *Fas* gene (Exon 4) of lymphocyte belongs to healthy, ALL, and CLL patients. Bands were fractionated by electrophoresis on a 1.5 % agarose gel (2 h., 5V/cm, 1X Tris-acetic buffer) and visualized under U.V. light after staining with ethidium bromide staining. lane M:100bp ladder. Lane:1.(Healthy), Lane: 2.(ALL), Lane:3.(CLL).

After alignment of *Fas* gene of the healthy, ALL and CLL groups with the *Fas* of *Homo sapiens* from the Gene Bank using the BioEdit software, we found that part of *Fas* gene (flank DNA sense and antisense of the gene) from healthy having 100% compatibility with standard *Fas* gene obtained from Gene Bank as shown in figure (6).

A: Sense of the partial *Fas* gene.

Score = 204 bits (110), Expect = 2e-50, Identities = 110/110 (100%), Gaps = 0/110 (0%), Strand=Plus/Minus.

```
Query 52  TTGGTGCAAGGGTCACAGTGTTCACATACAGTAGAGTTACAAAAAAAGTTTGGTTTACAT 111
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 789 TTGGTGCAAGGGTCACAGTGTTCACATACAGTAGAGTTACAAAAAAAGTTTGGTTTACAT 730

Query 112 CTGCACTTGGTATTCTGGGTCCGGGTGCAGTTTATTTCCACTTCTAAGCC 161
          ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 729 CTGCACTTGGTATTCTGGGTCCGGGTGCAGTTTATTTCCACTTCTAAGCC 680
```

B: Antisense of the partial *Fas* gene.

Score = 204 bits (110), Expect = 9e-52, Identities = 110/110 (100%), Gaps = 0/110 (0%), Strand=Plus/Plus.

```
Query 61  GGCTTAGAAGTGGAAATAAACTGCACCCGGACCCAGAATACCAAGTGCAGATGTAAACCA 120
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 680 GGCTTAGAAGTGGAAATAAACTGCACCCGGACCCAGAATACCAAGTGCAGATGTAAACCA 739

Query 121 AACTTTTTTTGTAACTCTACTGTATGTGAACACTGTGACCCTTGCACCAA 170
          ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 740 AACTTTTTTTGTAACTCTACTGTATGTGAACACTGTGACCCTTGCACCAA 789
```

Figure (6): Sequencing of sense and antisense flanking the *Fas* gene for healthy as compared with standard *Fas* obtained from Gene Bank (A: Sense of the gene; B: Antisense of the gene).

Morever, we also found that *Fas* gene (flank DNA sense and antisense of the gene) ALL and CLL obtained from patients having 100% compatibility with standard *Fas* gene of Gene Bank as shown in figure (7).

A: Sense of the partial *Fas* gene.

Score = 204 bits (110), Expect = 2e-50, Identities = 110/110 (100%), Gaps = 0/110 (0%), Strand=Plus/Minus.

```
Query 52  TTGGTGCAAGGGTCACAGTGTTCACATACAGTAGAGTTACAAAAAAAGTTTGGTTTACAT 111
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 789 TTGGTGCAAGGGTCACAGTGTTCACATACAGTAGAGTTACAAAAAAAGTTTGGTTTACAT 730

Query 112 CTGCACTTGGTATTCTGGGTCCGGGTGCAGTTTATTTCCACTTCTAAGCC 161
          ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 729 CTGCACTTGGTATTCTGGGTCCGGGTGCAGTTTATTTCCACTTCTAAGCC 680
```

B: Antisense of the partial *Fas* gene.

Score = 204 bits (110), Expect = 9e-52, Identities = 110/110 (100%),

Gaps = 0/110 (0%), Strand=Plus/Plus.

```
Query 61  GGCTTAGAAGTGGAAATAAACTGCACCCGGACCCAGAATACCAAGTGCAGATGTAAACCA 120
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 680 GGCTTAGAAGTGGAAATAAACTGCACCCGGACCCAGAATACCAAGTGCAGATGTAAACCA 739

Query 121 AACTTTTTTTGTAACTCTACTGTATGTGAACACTGTGACCCTTGCACCAA 170
          ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 740 AACTTTTTTTGTAACTCTACTGTATGTGAACACTGTGACCCTTGCACCAA 789
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Figure (7): Sequencing of sense and antisense flanking the *Fas* gene (Exon4) for ALL and CLL as compared with standard *Fas* obtained from Gene Bank (A: Senses of the gene; B: Antisense of the gene).

Although the results did not detect any mutations in exon 4 of *Fas* gene, other investigators have detected a lack of 20 base pair at Exon 9 resulting in a frame shift mutation which resulting the generation of a pre mature stop codon at amino acid 239 of Acute T-cell leukemia (ATL) (22 & 23), specify deletions in exon 9 in *Fas* gene, five missense mutations and one silent mutation in all 65 human non small cell lung cancers using PCR and DNA sequencing, they found that changes lead to loss of cells apoptotic functions and contribute to the pathogenesis of some human lung cancer. A novel *Fas* mutation which predicted the truncation of the intracytoplasmic domain of the Fas receptor in two siblings and the loss of *Fas* antigen expression by skipping of exon 4 of the Japanese patients (lymphoproliferative disorder) were detected (24) and Point mutation that was present in the splice acceptor site of intron 3 of the *Fas* gene were detected previously (18), this mutation results in the skipping of exon 4 and the complete loss of Fas expression.

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