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Sequencing of *IL-10* Gene Promoter for -592 (A/C) and -1082 (A/G) Positions in Iraqi Children Patients with Type 1 Diabetes Mellitus

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Abstract:
We studied the relationship between DNA sequencing of interleukin-10 (*IL-10*) gene promoter for -1082 (A/G) and -592 (A/C) positions with the concentration of IL-10 in blood serum of Iraqi children with type 1 diabetes mellitus (T1D). Fifty blood serum samples collected from children with age ranged between 7-12 years. Thirty-five blood samples collected from patient children with T1D, and compared with 15 healthy children age matched as control sample. The results revealed decreasing in anti-inflammatory IL-10 concentration in T1D patient's blood serum (0.068 Pg/ml) as compared with the control sample (0.111 Pg/ml). No significant differences were found in interleukin concentration between the studied samples when they analyzed with the Mann-Whitney U test. The DNA sequences of the *IL-10* gene promoter in 12 selected samples were recorded many gene mutations with addition, deletion and substitution types in all of the studied samples for the both -592 (A/C) and -1082 (A/G) positions. The 12 sequenced PCR products were submitted in the NCBI gene bank. The accessions numbers of these submitted sequences started from KX138250 to KX138261. In conclusions, low or absent concentration levels of IL-10 was found in T1D patient blood serums. Also, many gene mutations were detected in *IL-10* gene promoter for the studied sequences of T1D patients as compared with the same gene promoter which published in NCBI. These mutations maybe related with no or low concentration of *IL-10* in T1D patient serums.
Keywords: IL-10 gene, diabetes mellitus Type1, Promoter, IL-10, -592 (A/C) and -1082 (A/G) gene positions.

1.Introduction

Medical The produces either of the defect in the secretion of the hormone insulin or insulin reaction or both was known as metabolic disorders group ends with hyperglycemia [1]. Diabetes mellitus is caused by the inability of the β cells for the production of insulin in sufficient quantity or stopped production definitively for this is known as insulin-dependent diabetes mellitus or when the body is unable to use such material effectively and this is called diabetes mellitus (T2D), or non-insulin dependent diabetes mellitus [2]. Diabetes mellitus (T1D) is caused by the loss of immune regulation and lead to hypo-responsiveness of the T cells (Th2), and activation of Th1 cells [3]. Th2 was induce the component of anti-β cell immunity and mediated principally by IL-10 [4]. Interleukin-10 molecular weight is 17-20 kda [5]. Many researches since the discovery of IL-10 in the 1980s have led to its recognition as a pleiotropic immune-modulatory cytokine that affects both the innate and adaptive immune systems. IL-10 is produced by a wide range of cell types, and characterized by its ability to inhibit the inflammatory response by targeting certain cells, such as macrophage, Neutrophils, Eosinophil's and mast cells, Th2, Macrophages, Monocytes, and CD4+CD25+T cells. They have receptors reflect on hematopoietic and non-hematopoietic cells are IL-10R1 and IL-10R2 [5, 6]. Interlukin-10 represent as a mediator of suppression used by T regulatory cells [7]. Cytokines involved in urging further injury and diabetes T1D direct role through

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effective cell cytotoxic T and the indirect role through the effectiveness of cell by pro-inflammatory. Cytokines represent a centrist role in regulating the immune response and the response depends on several factors such as injury inflammation and the effects of hormones and has a relationship with gene polymorphisms [8]. The pathogenesis of diabetes mellitus – related to mediator IFN-γ Produced by the Th1 cells, while protection has to do with Th2 cells that produce cytokines Interlukin-10 and Interlukin-4 [9, 10,11]. Interlukin-10 is pleiotropic and has different effects in most types of hematopoietic cells [5]. Interlukin-10 encoding by gene located on chromosome 1 (1q31–1q32) [12, 13, 14]. There are 75% of the variation was genetically determined in *IL-10* gene [15]. Several polymorphic sites have been described within the promoter region, including two microsatellite polymorphisms and three bi-allelic polymorphisms positions -1082 (A/G), -819, and -592 (A/C) from the start site of the transcription [13, 16, 17]. Three functional SNPs in the *IL-10* gene locus promoter at –1082 (A/G), -819 (C/T) and -592 (A/C) from the transcriptional start site have been confirmed, and there are indications that they influence transcription of *IL-10* gene [18]. The aim of this study is to found the reasons of the absent or the low concentrations of Interlukin-10 production in diabetes mellitus (T1D) patients by using DNA sequencing of *IL-10* promoter gene region for the -592 (A/C) and -1082 (A/G) positions.

2.Materials and Methods

2.1.Subjects

Thirty-five patients with diabetes mellitus T1D (18 males and 17 females) and 15 healthy (control) persons were enrolled in the work. All subjects were obtained from Central Teaching Hospital of Pediatric and Al-Mustansiriya University National Diabetes center in Baghdad-Iraq. The disease T1D was diagnosed international criteria, by the consultant medical staff at these hospitals. The age range 7-12 years in all cases and controls. Collecting of the peripheral blood was done by using EDTA tubes to prevent anticoagulants and the isolation of DNA was performed from the collected blood.

2.2.Detecting of Interleukin-10

Two and half ml of patients and healthy peripheral blood samples was used for detecting Interlukin-10. Serums were obtained by centrifugation of blood samples at 1000 rpm for 10 min. Interleukin-10 ELISA development kit (PepRotech company-UK) was used for detecting Interlukin-10 by using ELISA instrument (Bio tech, USA).

2.3.Genotyping

Extracted DNA from venous blood by using ReliaPrepTM Blood gDNA Miniprep System kit (Promega) are used for the genetic analysis. The promoter of *IL-10* gene at position – 592 and -1082 (A/G) were genotyped using the polymerase chain reaction (PCR) approach. Primers for this position was used depending on [19], and synthesized in Alpha DNA company (Canada). Primer sequences of the promoter of *IL-10* gene at both positions with approximately 315 bp products size were show in Table (1).

Table 1. Primers for genotyping promoter of *IL-10* gene at -590 and -1082 (A/G) positions.

Primer	Sequence (5′ → 3′)	Product size (bp)
IL-10 −592 (F)	5′-TGCAGACTACTCTTACCCACTTCC-3′	315
IL-10 −592 (R)	5′-AATAATTGGGTCCC CCCAAC-3′	
IL-10 −1082 (F)	5′-GACAACACTACTAAGGCTcCTTTGGGA-3′	315
IL-10 −1082	5′-GTGAGCAAACTGAGGCACAGAaAT-3′	

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 8197 - 8206
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2.4.Polymerase chain reaction (PCR)

PCR approach was used for amplifying the promoter of *IL-10* gene at –590 and -1082 (A/G) positions. Go Taq® Green Master Mix (2X) (Promega, USA) was prepared depending on [19]. DNA template and primers were added to PCR Master Mix tubes. The final volume for PCR reaction was made up to 25 µl with nuclease-free water (Table 2). Thermocycler (Esco, Singapore) instrument was used and the reaction mixers was placed in. Conditions of PCR reaction for the all reaction mixers were illustrated in Table (3).

Table 2. PCR mix reaction for detecting of *IL-10* gene promoter at -590 and -1082 (A/G) positions.

Component	Volume (µl)	Final concentration
Go Taq® Green Master Mix (2X)	12.5	1X
Each primer (Foeward+Reveres)	2	1 µM
DNA template	2	100 ng
Nuclease-Free wate	6.5	-
Final volume	25	-

Table 3. Conditions of PCR reactions for detecting promoter of *IL-10* gene at -590 and -1082 (A/G) positions.

Steps	**Temperature (ºC) and Cycles**		**Time**
Template denature	94		5 min
Initial denaturation	94	30 Cycles	15 Sec
Annealing	55		15 Sec
Extension	72		30 Sec
Final Extension	72		5 min
Incubation	4		5 min

The PCR Products were resolved on agarose gels (1.5%). The DNA ladder (100 bp) (Bioneer, Korea) was loaded on the agarose gel. Two microliter of Bromophenol blue dye was loaded with all reaction mixer samples. The gel electrophoresis performing was done by using 75V for 3 hrs. Ethidium bromide (Promega, USA) was used for staining the agarose gel for 30 min. Documentation of the gel was done by using gel documentation system (Biocom, USA).

2.5.DNA sequencing

PCR products for twelve samples were subject to DNA sequencing depends on the concentration levels of Interlukin-10 in both patients and healthy samples. AccuPrep®PCR Purification kit (Bioneer Corp., Korea) was used for PCR products purification before sequencing approach. DNA sequencing was done by using genetic analyzer 3500 instrument (Applied Biosystems, USA). The used protocol was BDxStdSeq50_POP7_1, BigDye terminator V3.1, which provided by Applied Biosystems

2.6.Statistical analysis

Statistical Package for Social Sciences ver. 22 (SPSS) was used for data analyzing. Frequencies percentage and significant differences data between diabetes mellitus T1D patients and controls samples were given by Mann-Whitney U test at $P<0.05$. DNA sequencing was analyzed by using two specific programs; Chromas Pro. version 1.6, 2012 (Technelysium Pty Ltd) and BioEdit Sequence Alignment Editor [20].

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3.Results and Discussion

3.1. Interlukin-10 concentrations

Rats The results revealed decreasing of Interlukin-10 concentrations in diabetes mellitus (T1D) patient's serum (0.068±0.011 Pg\ml) as compared with the healthy children (0.111±0.031 Pg\ml). No significant differences were found when Mann-Whitney U test was used at P<0.05 (Table 4).

Table 4.Concentrations of Interlukin-10 in diabetes mellitus (T1D) patients and healthy children serum.

Interleukin	Patients (no. 35)	Confidence Intervals 95%		Healthy (no. 15)	Confidence Intervals 95%		Probability (P<0.05)
	Mean ± S.E (Pg\ml)	Higher value	Lower value	Mean ± S.E (Pg\ml)	Higher value	Lower value	
10	0.068±0.011	0.066	0.002	0.111±0.031	0.126	0.004	0.618

Table The reason of decreasing of Interlukin-10 concentration levels in patients in this study is not clear, and maybe there is a correlation between the diabetes mellitus (T1D) and the Interlukin-10. No *IL-10* gene expression study was done to support the lower concentration levels of Interlukin-10 in patient children. The results of this study agree with some other studies, which found that the concentrations of IL-10 and IL-4 in blood serum of patients with diabetes mellitus (T1D) were decreased, while the concentrations of IL-17 and INF-γ were increased [21, 22, 23]. Also, [24] was studied the concentration levels of IL-10 in diabetes mellitus T2D patients in Poland. This study results revealed that the concentration of IL-10 in the patient serum concentrations was lower in comparison with the healthy people, but [5] found within European increasing of IL-10 levels in diabetes mellitus (T1D) patients as compared with healthy persons. The study of cytokines profile in newly diagnosed children with diabetes mellitus (T1D) divided by age into two categories (>10 years and <10). Higher levels of IL-10 in serum were observed in the investigated patients <10 years compared to controls). The statistical analysis revealed a significant difference between patients and controls. The mean levels of serum Interlukin-10 were also significantly elevated in >10 years old patients than controls [26]. [27] was studied cytokines levels in 56 Iraqi patients with diabetic mellitus (T1D) ranging age from 2 to 29 years. His study results showed no significant differences in mean concentration levels of IL-10 in patients when compared with the control sample, while [28] found significant differences in mean concentration levels of Interlukin-10 between patients with diabetic mellitus (T1D) and healthy persons. The mean level concentration of Interlukin-10 was studied on 71 patients with diabetic mellitus (T1D). The results revealed low concentration levels of Interlukin-10 in patients as compared with control [29]. Higher concentration levels of Interlukin-10 with significant differences was found in 35 Chinese patients (age 3-14 years) with diabetic mellitus (T1D) in comparison with the control [30].

3.2. Promoter of IL-10 gene

The promoter of *IL-10* gene at -592 (A/C) and -1082 (A/G) positions were detected in all diabetes mellitus (T1D) and healthy children samples. One band was appeared in each sample with approximately 315 bp molecular size when PCR technique was used (Figure 1).

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Received 25 April 2021; Accepted 08 May 2021.

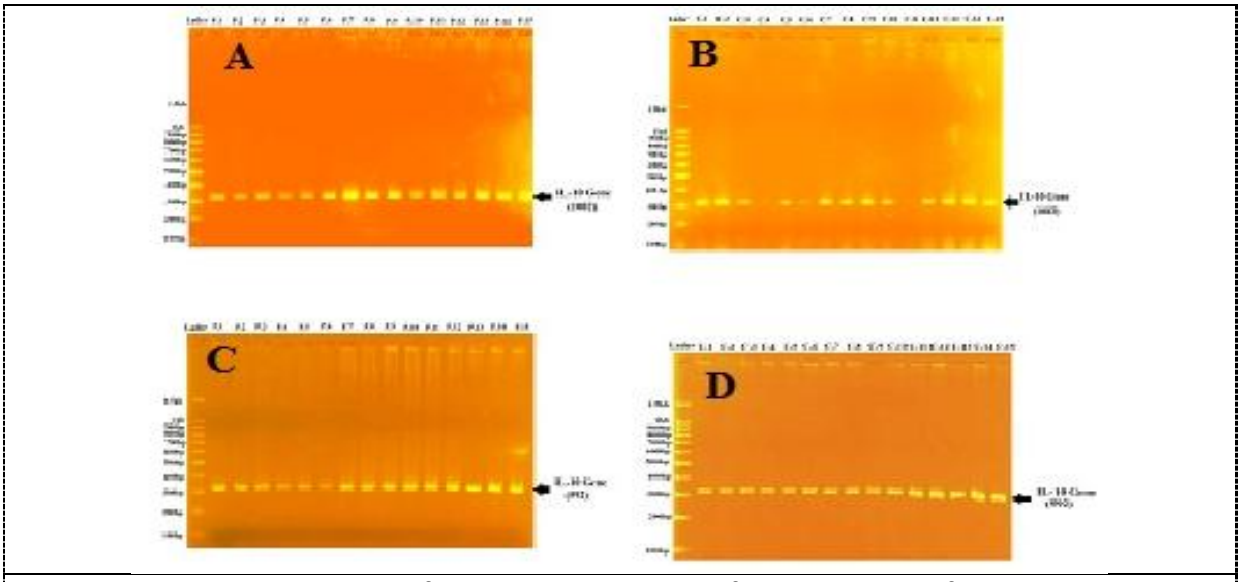

Figure 1. Gel electrophoresis for the PCR products of the promoter of *IL-10* gene in some patients and healthy children samples. A: promoter of *IL-10* gene (-1082 (A/G)) in patients with diabetes mellitus T1D, B: IL- promoter of 10 gene (-1082 (A/G)) in Healthy children, C: promoter of *IL-10* gene (-592 (A/C)) in diabetes mellitus (T1D) patients, D: *IL-10* gene promoter (-592 (A/C)) in Healthy children. Gel electrophoresis was done by using agarose gel (1.5%) concentration, 75 Volt for 2 hours. One band was appeared in each sample with approximately 315 bp molecular size.

3.3. Selected samples for DNA sequencing

Some selected samples of patients and healthy samples with different concentrations of Interlukin-10 were subject to DNA sequencing depended to Interlukin-10 concentrations (Table 5). The sequencing was done to explore the mutations or diversity in the nucleotides, especially in *IL-10* gene promoter which is important for RNA transcription.

Table 5. Some of the selected samples for DNA sequencing depended on Interlukin-10 concentrations.

Clone Id. (Patients)	IL-10 concentrations in patients (Pg/ml)	Clone Id (Healthy)	IL-10 concentrations in Healthy (Pg/ml)
PDM1 (-1082 (A/G))	0.09	HDM1 (-1082 (A/G))	0
PDM2 (-1082 (A/G))	0	HDM2 (-1082 (A/G))	0
PDM3 (-1082 (A/G))	0.02	HDM3 (-1082 (A/G))	0.281
PDM4 (-592 (A/C))	0	HMD4 (-592 (A/C))	0.173
PDM5 (-592 (A/C))	0.129	HMD5 (-592 (A/C))	0
PDM6 (-592 (A/C))	0	HMD6 (-592 (A/C))	0.093
Mean*	0.068	-	0.111

*= The mean of IL-10 concentrations is calculated for the all studied samples (Table 4).

3.4. DNA sequencing of IL-10 gene promoter at positions -1082 (A/G) and -592 (A/C)

Two Refseq were used for comparisons with the sample sequences, which founded in the NCBI. The first accession number for chromosome 1 is NC_000001.11 and the second accession number for the promoter of *IL-10* gene is AB098711.1. The results of DNA sequencing for PCR products are revealed many mutations with addition, deletion and substitution nucleotides when these sequences were compared with Refseq of *IL-10* gene. Analysis of sequencing results of *IL-10* gene promoter (-1082 (A/G) position) in patient samples showed addition of Thymine (T) and Guanine (G) in 1093 and 1172 positions and deletion of nucleotide sequences AAT in the 1215-1217 positions and deletion of ACAGG sequences in 1308-1312 positions (Figure 2-A). The healthy children samples showed no

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nucleotide addition. Deletion of Cytosine (C) and Adenine (A) nucleotides in the 1093 and 1172 positions was founded. Also, deletion of TTCTCA sequences in 1278-1283 positions and deletion of GG sequences in 1309-1310 positions were founded (Figure 2-B). The results revealed many nucleotides substations with 78 nucleotides substitution in patient in comparison with 51 nucleotides substitution in healthy samples. The highest ratio rate of substitution of T→C was founded in patients with 17.95% and the lowest ratio rate was 2.56% with T→G substitution. The healthy samples have highest ratio rate of substitution of G → T was founded in patients with 23.53% and the lowest ratio rate was 1.96% with C→G substitution (Table 6). TATA box was absent in the patient and healthy sequence samples. The TATA box is found at the binding site of RNA polymerase II and it binds to the transcription factor (TFIID).The sequencing results of *IL-10* gene promoter (-592 (A/C) position) in patient samples showed addition of Thymine (T) in 1402 position and deletion of nucleotide sequences ACAGCT in the 1464-1468 positions (Figure 2-C). Deletion of Guanine (G) and Cytosine (C) also founded in the 1488 and 1489 positions, respectively. The healthy children samples showed addition of G nucleotide in the 1303 position with deletion of T, Adenine (A) and G nucleotides in 1286, 1334 and 1335 positions, respectively. Also, deletion of TAGA nucleotides in the 1352-1355 positions was founded (Figure 2-D). The results revealed many nucleotides substations with 57 nucleotides substitution in patient in comparison with 66 nucleotides substitution in healthy samples. The highest ratio rate of substitution of G→A was founded in patients with 17.54% and the lowest ratio rate was 1.75% with A→T substitution. The healthy samples have highest ratio rate of substitution of C→T was founded in patients with 15.15% and the lowest ratio rate was 1.52% with G→C substitution (Table 6). TATA box was absent in the patient sequence samples. These sequences are submitted to the NCBI and given accession number started from KX138250.1 and ended with accession number KX138261.1 (Table 7).

Figure 2. DNA sequencing of *IL-10* gene promoter in some diabetes mellitus (T1D) patients and healthy selected samples.

A: promoter of *IL-10* gene at -1082 (A/G) position in patients with T1D, B: promoter of *IL-10* gene at -1082 (A/G) position in healthy children, C: promoter of *IL-10* gene at -592 (A/C) position in patients with T1D, D: promoter of *IL-10* gene at -592 (A/C) position in healthy children. Arrows refer to deletion sequences. NC_000001.11 is accession number for chromosome 1. AB098711.1 is accession number for *IL-10* gene promoter. Arrows refers to deletion nucleotides.

Table 6.Nucleotides substitution in promoter of *IL-10* gene at positions -1082 (A/G) and -592 (A/C) in diabetes mellitus (T1D) patients and healthy children samples.

Substitution	Patients with T1D		Healthy children	
type	-1082 (A/G)	-592 (A/C)	-1082 (A/G)	-592 (A/C)

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 8197 - 8206
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	No.	%	No.	%	No.	%	No.	%
A → C	3	3.85	3	5.26	2	3.92	4	6.06
G → A	7	8.97	10	17.54	6	11.76	14	21.21
A → G	10	12.82	6	10.53	5	9.81	5	7.58
C → T	11	14.10	8	14.04	2	3.92	10	15.15
T → C	14	17.95	6	10.53	5	9.81	8	12.12
A → T	6	7.69	1	1.75	5	9.81	-	-
G → T	8	10.26	4	7.02	12	23.53	8	12.12
C → G	4	5.13	3	5.26	1	1.96	2	3.03
T → G	2	2.56	4	7.02	4	7.84	2	3.03
C → A	4	5.13	7	12.28	-	-	4	6.06
G → C	6	7.69	2	3.51	3	5.88	1	1.52
T → A	3	3.85	3	5.26	6	11.76	8	12.12
Total	78	100	57	100	51	100	66	100

Table 7. Accession numbers of *IL-10* gene promoter at positions -590 and -1082 (A/G) in diabetes mellitus (T1D) patients and healthy samples.

Sequence ID	Clone ID	Accession number (NCBI)
***IL-10* -1082 (A/G) in healthy children**	HDM1	KX138250.1
***IL-10* -1082 (A/G) in healthy children**	HDM2	KX138251.1
***IL-10* -1082 (A/G) in healthy children**	HDM3	KX138252.1
***L-10* -1082 (A/G) in patient children with diabetes mellitus (T1D)**	PDM1	KX138253.1
***IL-10* -1082 (A/G) in patient children with diabetes mellitus (T1D)**	PDM2	KX138254.1
***IL-10* -1082 (A/G) in patient children with diabetes mellitus (T1D)**	PDM3	KX138255.1
***IL-10* -592 (A/C) in healthy children**	HMD4	KX138256.1
***IL-10* -592 (A/C) in healthy children**	HMD5	KX138257.1
***IL-10* -592 (A/C) in healthy children**	HMD6	KX138258.1
***IL-10* -592 (A/C) in patient children with diabetes mellitus (T1D)**	PDM4	KX138259.1
***IL-10* -592 (A/C) in patient children with diabetes mellitus (T1D)**	PDM5	KX138260.1
***IL-10* -592 (A/C) in patient children with diabetes mellitus (T1D)**	PDM6	KX138261.1

This study was deal with *IL-10* gene promoter sequencing, especially in Iraqi children with diabetes mellitus (T1D). Many gene mutations were found when the studied sequences were compared with Refseq which published in NCBI. These mutations maybe related with no or low concentration of Interlukin-10 in patients with T1D. The promoter of *IL-10* gene has correlation with RNA polymerase II and this have role in mRNA transcription. So, any defect in this promoter region maybe reflects to Interlukin-10 mRNA production or maybe due to the differences in the Iraqi genome. No genomic studies were done on Iraqi population to support these results. A genetic defect in the *IL-10* gene is lead to the development of the immune disease and leads to occurrence of chronic disease [31]. A mutation is defined as any change in a DNA sequence away from normal. In contrast, a polymorphism is a DNA sequence variation that is common in the population. We don't find other studies deal with the mutations detecting in *IL-10* gene promoter and the production of *IL-10* in blood serum of immune disease. Previous studies have investigated several polymorphic sites in this region specially at -592 (A/C), -819 (C/T) and -1082 (A/G) positions in different immune diseases. A

Caucasians and Asians population were found to be different in genotype frequency of *IL-10* polymorphisms. Relationship was found between cytokine production and the allelic polymorphisms of cytokine genes [32, 33]. Single nucleotide polymorphism (SNP) genotype and haplotype frequencies of *IL-10* Promoter gene appear to exhibit different distribution according to ethnicity [17, 34, 35]. These polymorphisms might configure several different haplotypes, but three only, ATA, ACC and GCC, have been described in the populations of Caucasian. The synthesis rate of *IL-10* mRNA and ultimately cytokine production depend on the final gene configuration[13, 36]. A minor role of *IL-10* gene in the autoimmune diabetes risk was proved, although the same association trend with IL-10G*12 allele as was previously observed for multiple sclerosis and rheumatoid arthritis [17], while in the other studies which done by [19] and [37] showed that the polymorphism of *IL-10* gene mutant -1082 (A/G), -819 (C/T) and -592 (A/C) did not revealed any significant differences, which link the T1D with these mutations and these mutations play a protective role from infected by this disease. The polymorphisms in promoter of *IL-10* gene maybe have a role in T1D infection [19]. Also, [38] suggest a strong role between the -1082 (A/G) mutant in promoter of *IL-10* gene and T1D in *IL-10* gene Polymorphisms in promoter region are associated with susceptibility to or clinical presentation of T1D. [39] was study the polymorphisms frequency of -1082 (A/G), -819 (C/T), and -592 (A/C) mutants in Japanese patients with T1D and in healthy control subjects in a case-controlled study. The polymorphism frequencies of allelic and haplotypic in the *IL-10* gene promoter region were similar in T1D patients and in control samples. The -819T and -592 (A/C) allele were associated with adult-onset (>18 years) of the disease. These results by these researchers suggest that the promoter of *IL-10* gene polymorphisms are associated with the age in Japanese T1D patients.

4. Conclusions

Low or absent concentration levels of IL-10 was found in T1D patient blood serums. Also, many gene mutations were detected in *IL-10* gene promoter for the studied sequences of T1D patients as compared with the same gene promoter which published in NCBI. These mutations maybe related with no or low concentration of IL-10 in T1D patient serums

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