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Evaluate IL-10 level and gene polymorphism in β -Thalassemia patients with related to microbial infection and disease outcome

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Abstract--Thalassemia is a genetic blood disorder inherited from the parent with unusual production of hemoglobin, infectious complications, immune abnormalities and iron overload with related to organ damage that consider a chief cause of morbidity and mortality. So this study aimed to evaluate the important causative agents and the role of IL-10 gene polymorphism in progressive disease. A Case-control study was performed to 46 Beta-thalassemia patients attended to inherited blood center in AL-Zahraa teaching hospital with group of 30 healthy individual as control. Blood sample was collected from all patients and control. IL-10 level were measured by enzyme-linked immunosorbent assay (ELISA). Singule nucleotide polymorphism detected by ARMS-PCR technique. The result shown that 46(46%) patients with thalassemia, the male higher than female with frequency (65%), according to infection 32(70%) were TTI with HCV a most common pathogen 19(41%). This study explain that serum level of IL-10 is significantly higher among patients with thalassemia (98 ± 27.53) pg/ml compared to healthy control (10.67 ± 1.49) pg/ml and was higher in HCV infected patients (121.56 ± 32.60) pg/ml than non-infected with HCV record (73.61 ± 18.29) pg/ml. The genotyping of IL-10 revealed three genotypes; AA, AG and GG types with frequency (32.6%, 28.3% and 39.1%) respectively in thalassemia patients, and the GG/allele G is the risk factor in thalassemia patients that infected with HCV (52.6%) while patients with non-infected HCV (18.5%).

Keyword: transmission, transfusion, infection, IL-10, HCV.

Introduction

B-Thalassemia is one of the most common groups of hereditary haemoglobinopathies. β Thalassemia major is a public health problem worldwide that depended on regular blood transfusion which on the long term leads to iron overload and complications in multiple organs, enhanced susceptibility to infections as a consequence of coexistent immune deficiency (Zarghamian *et al.*, 2020). Thalassemia affecting approximately 3% (1.5 million) of the population and resulting in serious health problems such as increased morbidity, premature death (Riaz *et al.*, 2022). Viral agents such as hepatitis B and C infection can cause a decrease in glomerular filtration rate (GFR) of thalassemia patients (Hashemieh *et al.*, 2020). The Interleukin IL-10 is a 36 kDa homodimeric anti-inflammatory cytokine expression in T cells, B cells, monocytes cells and NK cells, IL-10 exhibit a wide array of immunosuppression activities on various immune cells, among these the inhibition of antigen presenting cell function, inhibition of expression of inflammatory cytokines, inhibition of cytotoxic T cell (CTL) responses and induction and regulatory T cells, and regulation of the survival and proliferation of B cells (Saraiva., 2020). The IL-10 gene is located on chromosome 1q31–32. The promoter spans a region 5 kb upstream of the transcription start site, and both this area and the gene itself are known to contain several polymorphisms (Mocellin., 2021).

Promoter region polymorphisms appear to be correlated with variations in transcription, three of several polymorphic sites within the promoter region of *IL-10* have been described in some detail, these are -1082 A to G substitution (rs1800896) where A is the ancestral allele that lie within a putative negative regulatory region that is a binding site of Ets transcription factors and STAT-3 according to the National Center for Biotechnology Information (NCBI) SNP database (Mormann *et al.*, 2014). A large number of association studies have found that polymorphisms of *IL-10* are involved in the susceptibility to many diseases, such as asthma (Kadhem and Darweesh, 2017), Thalassemia (Surhan *et al.*, 2018), recurrent aborted women with viral infection (Hate and Darweesh, 2019).

Patients and Method

Patients and control characterization

A case –control study was conducted during October 2021-febreuary 2022 in AL. Zahra teaching hospital in AL-Najaf provenances on 46 patients suffering from thalassemia with age range (1 -39 years). In addition to 30 randomly healthy persons (20 male and 10 female) with age range between (1-60) years.

Collection of sample

Five ml of blood specimens were collected from all subjects, 4 ml collected in EDTA tube from which 2 ml centrifuged to separate plasma for viral RNA for HCV and HBsAg identification by Anti-HCV(CLIA) and HbsAg(CLIA) kite(China) using Snipe Maglumi 1000 Fully Automated machine and (2 ml) for extracted DNA by (favorgen kite/ china) to detected IL-10 gene polymorphism according

to ARMS-PCR assay while (1 ml) collected in gel tube to separate serum then stored at -20°C until used for measuring IL- 10 according to (Solarbio \ China). Urine used to isolate bacterial species that inoculated on general and differential media.

DNA isolation and PCR

Genomic DNA was extracted from fresh peripheral blood (2 ml in EDTA) using a commercially available kit according to the protocol of favorgen/china and stored at -20 C till use. Single nucleotide polymorphisms (SNPs) related to the IL-10 (-1082) were determined using PCR with Amplification Refractory Mutation-Polymerase Chain Reaction (ARMS-PCR) in two reactions employing one common forward and two reverse primers 5'- AGCAACACTCCTCGTCGCAAC-3' (forward) ,5'-CCTATCCCTACTTCCCCC-3 (reverseone) and 5'-CCTATCCCTACTTCCCCT3' (reverse tow) with an amplicon size of 179 bp. The reaction mix was done in 25µl volumes include 5µl of template DNA, GoTaq ®Promega Green Master Mix 2X 12.5 µl, Primers (foreword 2 µl and reverse 2 µl) and Nuclease Free water 3.5 µl (Applied PCR system, USA).and PCR conditions for *IL0* gene are initial denaturation at 94°C for 5 min, followed by denaturation at 94°C for 30s,annealing at 60°C for 1 min and 1 min of extension at 72°C, with afinal extension of 7 min at 72°C .The resultant PCR products were resolved by electrophoresis(UV - Trans illuminator) on 1g agarose gel stained with 2 µl (0.5 % concentration) from ethidium bromide, the run lasted for 1 hour for 100 V. The gel was then photographed (digital camera) on UV light and scored for the presence or absence of an allele specific band. The following samples were excluded: samples from individuals who had received antibiotic therapy; patient not fill all information or correct sample patients suffering from other type of thalassemia

Ethical committee

This study was approved by the Ethics Committee of the Faculty of Science, Kufa University (NO 17985) and Medical Ethics Committee of the Ministry of Health in Iraq.

Statistics

Analysis of data was performed by using Statistical Package for Social Science (SPSS) system/ version 20. Results of ELISA expressed as mean $\pm$ S.E. The presence of certain genotype and disease status the odds ratio (OR) was used.. Differences of genotype and allele frequencies between thalassemia and control groups were also analyzed using the 2 test. Pearson coefficient and odds ratios (ORs) for the risk of thalassemia and their 95% condense intervals (CIs) were calculated using logistic regression analysis. All statistical analyses were performed by Microsoft excel and the Graph Pad software (prism version 6). The difference was considered significant, if $p < 0.05$.

Results and Discussion

Demographic study

Distribution of study subjects according to type of disease, age and sex

A study on 46(46%) patients with thalassemia from which 32(70%) were TTI and HCV a most common pathogen with 19(41.3 %) and 19.5% of patients infected with several types of bacteria such as *K.pneumonia* , *E.coli*, the male higher than female with frequency (65%). In addition to 30 apparently healthy subjects as controls group as shown in table 1.

Table 1
Distribution of study subjects according to type of disease, age and sex

Blood transfusion infection		
	Thalassemia N=46	Healthy group
Male	30 (65.2)	20 (66.7)
Female	16 (34.8)	10 (33.3)
Age Mean $\pm$ SE	18.60 $\pm$ 4.10	21.87 $\pm$ 6.36
Age range	1-39	1-40
1-9	15(32.6)	8(26.6)
10-19	12 (26)	10 (33.3)
20-29	8 (17.3)	8 (26.6)
30-39	1 (2.1)	4 (13.3)
HCV+	19(41.3)	-
HBV+	4(8.6)	-
Bacterial infection	9(19.5)	-
Non-infected patients	14(30)	

The result observed that male comprise 30 (65.2) and the age range 1-39 years with high frequency in age between 1-9 years 15(32.6) with mean age (18.60 $\pm$ 4.10). This result agree with (Ali and Abdulla., 2020) who found that male recorded 94(54%) and 81(46%) were females with a ratio 1.2: 1 and mean age of patients were (10.5 $\pm$ 5.8 years) , the range age of patients from 5 months to 34 years and found that more than half (55%) between aged 1 - 10 years. In study by Amin *et al.*, (2020)confirmed that male more susceptible to β -thalassemia disease with frequency 56.6% males and 43.4% females, with ratio male : female ratio of 1.3 : 1. The patients' ages ranged between 1.4 - 54 years with a median of 15 yrs.

According to HCV infection

The results of this study show 41.3% of thalassemia patients were anti HCV sera-positive .This in line with local study by (Albakaa *et al.*,2020) observed that male more than female with frequency 53.57 , their ages range from (2-34) years and HCV PCR positive were (57.14 %) while (Surhan *et al.*,2018) founded that 36.6% of thalassmeic patients sera was confirmed positive for anti- HCV-antibodies. In Iran (Aminianfar., 2017) reported that 0.7% of thalassemia patients were positive for HBV and 60 10.2% were positive for HCV. Dawood *et*

al., (2020) in the province of Mosul explain that Iraq is one of the countries in the Middle East that has had an increased incidence of Hepatitis C infections over the past few years, and the increasing incidence can be observed in hemodialysis and thalassemia patients, due to the lack of health awareness among individuals, the failure of follow-up of new cases, and the deficiency of appropriate treatment in addition to the failure invention suitable vaccine.

Immunological Study

Evaluation IL-10 serum level in Thalassemia patients and healthy group

This study explain that serum IL-10 level was significantly increased in thalassemia patients (98 ± 27.53 pg/ml in compare to healthy control group (10.67 ± 1.49) pg/ml . figure 1.

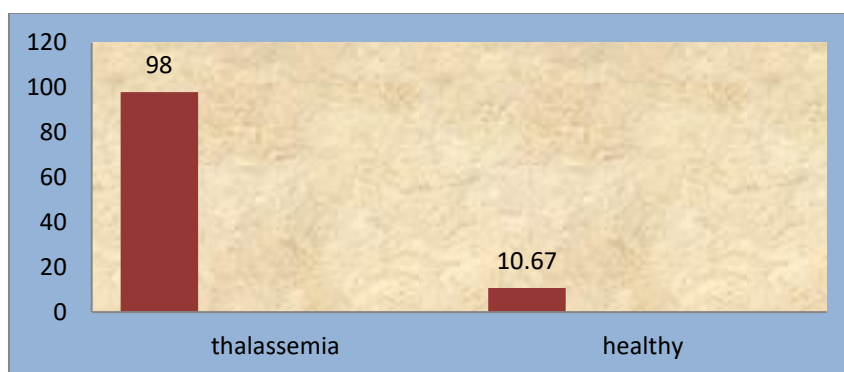


Figure 1. Illustrate the IL-10 serum level in thalassemia patients and healthy group

The result agrees with result by Salam and Mohsin (2021) that found a higher concentration of IL.10 serum level in thalassemic patients(22.532 ± 2.274) compared with control group(6.506 ± 0.906) $P < 0.0001$ and demonstrate that the concentration of IL.10 show a clear association between IL.10 and anemia. Balouchi *et al.*, (2014) showed a significant higher concentration of IL-10 ,TGF- β and IL-23 in the patient group than control group and concluded that several immunological abnormalities have been characterized in β -thalassemia which linked to cytokines . Surhan *et al.*, (2018) shown that IL-10 level is significantly increase in thalassemia patients(23.9 ± 4.4) than control(2.9 ± 1.5) $P < 0.0001$.

Evaluation IL-10 serum level in thalassemia patients according to splenectomy

This result explain that thalassemia patients appear highly decreased in IL-10 serum level after splenectomy than non –splenectomy patients (14.98 ± 2.34 , 179.54 ± 12.51 pg/ml) respectively as show in figure 2.

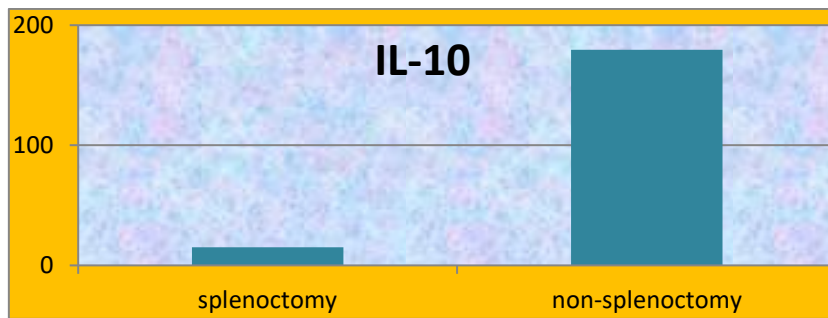


Figure 2. Illustrate the IL-10 serum level in thalassemia patients according to splenectomy and non splenectomy patients

This study in line with study by Shebl *et al.*, (2018) that found IL-10 serum level was significantly lower in post splenectomies thalassemia patients (258.155 ± 197.527) compared with non- splenectomies thalassemia patients (1366.500 ± 1565.521) and control group (1195.400 ± 1306.415), this is because multi-transfusions could be responsible for a change in the subset of circulating lymphocytes that could contribute to a state of partial immune deficiency in beta thalassemia patients which is more prominent among the splenectomies' patients (caligiuri *et al.*, 2005). showed that IL-10 production by splenectomies' patients are less than non- splenectomies' , also (Jison *et al.*, 2004) showed that IL-10 is a cytokine with potent anti-inflammatory activity which reduces the production of various cytokines including IL-1, IL-6, IL-8, IL-12, TNF- α and GM-CSF to promote uptake and retention of iron in the reticuloendothelial system so patients with iron overload have decrease in IL-10 as there is negative correlation between IL-10 and serum ferritin which reinforce the existence of a clear inflammatory state in patients with iron overload as a result of excess iron.

Estimation the level of IL-10 in thalassemia patients according to HCV infection

The present study recorded that thalassemic patients infected with HCV was 121.56 ± 32.60 while patients non infected with HCV record (73.61 ± 18.29) pg/ml . As show in figure 3.

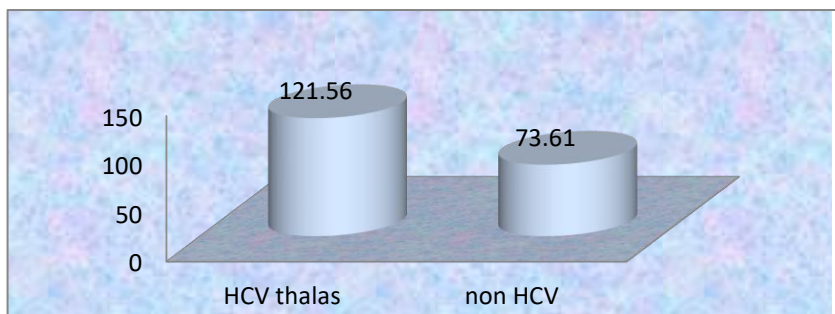


Figure 3. Illustrate the IL-10 serum level in thalassemia patients according to infected with HCV

This result is compatible with result obtained by Mahmoud *et al.*, (2018) that found a significant increase in the concentration of IL-10 in HCV patients (33.62 ± 7.3) as compare with thalassemia patients without liver hepatitis (8.74 ± 2.5), indicates the predominance of Th2 cytokine which promote the persistency of virus, also HCV core and NS3 induced production of the anti-inflammatory cytokines such as IL-10 that can lead to severe fibrosis in hepatitis C virus. Also this result is in line with result obtained by Surhan *et al.*, (2018) observed that the concentration of IL-10 in thalassemia patients with HCV infection was 36.1 ± 8.3 pg/ml, thalassemia patients without HCV infection was 13.1 ± 2.5 pg/ml and normal persons were 2.9 ± 1.5 pg/ml, $P < 0.0001$, this may be attributable to that chronic transfusion program will result in continuous antigenic stimulation and iron overload with consequent abnormality in cell mediated immunity such as reduce CD4\CD8 ratio, T-cell subset anomalies and alteration in T-cell number and function. Kane and Mosser., (2011) confirmed that increased IL-10 during infection down regulates many processes including IFN- γ production, which in turn reduces macrophage activation and disrupts the effective cellular response to clear the pathogen.

Molecular study

1IL-10 Gene "-1082 A/G" in Thalassemia patients

Distribution of IL-10 Gene "-1082 A/G" polymorphism in β -TM patients

The distribution of IL-10 -1082 A\G polymorphism was detected by ARMS-PCR technique, at this locus there are three genotype; AA, GA and GG with band sizes of 179bp. The genotypes frequency in thalassemia patients were as follow; AA(32.6%), GG(39.1%) and GA (28.3%); while in the control ;AA (76.6%) , GG (6.7%) and GA (16.7%), Table 2 and figure 4.

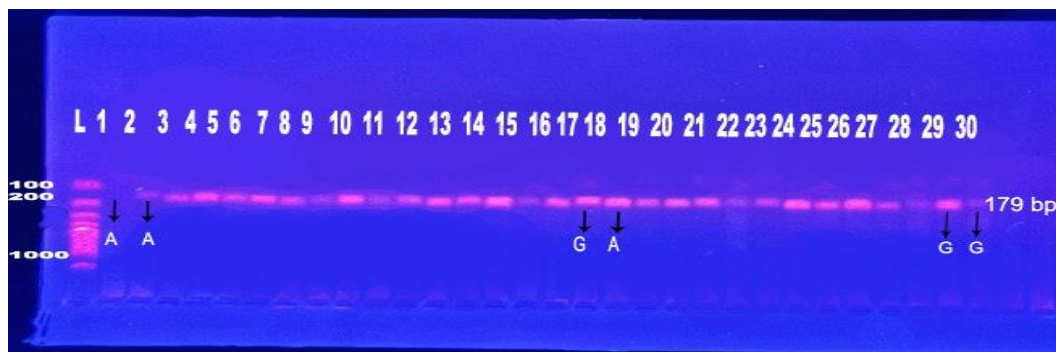


Figure 4. Ethidium bromide-stained agarose gel of PCR amplified 179bp of IL-10 gene in thalassemia patients. L (ladder), 3 (AA genotype), 9 (GA genotype), 3 (GG genotype), agarose gel 1%, 100 vol , 1 hour

In the present study the results show a significant difference in the genotype and allele frequency of IL-10 -1082G/A polymorphism between thalassemia patients and the control group. The GG genotype and G allele were overrepresented in patients compared to the control group. Also , it was observed that individuals with the GG genotypes had higher risk for developing thalassemia, thus revealing that patients were more susceptible to thalassemia disease.

Table 2
Allele frequency of the IL-10-1082 A\G promoter variant among thalassemia patients and control

IL-10	Thalassemia n=46	Control n=30	OR(95 % CI)	P-value
G/G	18 (39.1%)	2 (6.7%)	6.774 (1.4215 to 32.2830)	0.0163*
G/A	13 (28.3%)	5 (16.7%)	1.969 (0.5615 to 5.4546)	0.2500 ns
A/A	15 (32.6%)	23 (76.6%)	0.195 (0.0697 to 0.5495)	0.0020**
Allele frequency				
G allele	49	9	4.972 (2.1935 to 11.2736)	0.0001***
A allele	43	51	0.201 (0.0887 to 0.4559)	
*(P<0.05): significant , **or*** (P<0.05) higher significant				

Schaaf *et al.*,(2003) confirmed that IL-10 allele G homozygous patients had the highest risk for septic shock and the G allele, associated with high IL-10 release that influence the outcome of disease. Previous study found that the IL-10 polymorphism for low production by (AA genotype) were found to very significantly increase the risk of graft rejection in children with β -TM undergo bone marrow transplantation (Swiatek *et al.*,2012). Bagheri *et al.*,(2005) show presence of an "A" at -1082 and at -592 has been related to low IL-10 production in the cultured cells, and show that IL-10 haplotype A decreased significantly in thalassemic patients. Hoffmann *et al.*,(2001)show that both recipient and donor *IL-10* gene polymorphisms affect hematopoietic recovery, *IL-10 -1082A* which is associated with lower levels of IL-10 and presence of recipient *IL-10 -082G/G+G/A* genotype associated with higher and intermediate levels that correlated with delayed neutrophil and platelet recovery, respectively, this explain that GG genotype is risk factor in thalassemia patients.

Genotypes distribution of IL-10-1082 A\G among thalassemia patients according to the HCV

The result shows high frequency of GG (52.6%) than other haplotype AA (15.8%) and GA (31.6%) in thalassemia patients infected with virus compare with non-infected patients as shown in table 3.

Table 3
Correlation between genotype of IL-10 and infection with HCV in thalassemia patients

IL-10	HCV n=19	Non-HCV n=27	OR(95 % CI)	P-value
G/G	10 (52.6%)	5 (18.5%)	4.888 (1.3003 to 18.3812)	0.0188*
G/A	6 (31.6%)	7 (25.9%)	1.318 (0.3612 to 4.8138)	0.6754 ns
A/A	3 (15.8%)	15 (55.6%)	0.150 (0.0353 to 0.6382)	0.0102*
Allele frequency				
G allele	26	17	4.715 (1.9305 to 11.5194)	0.0007***
A allele	12	37	0.212 (0.0868 to 0.5180)	
*(P<0.05): significant , **or*** (P<0.05) higher significant				

This result is in line with local study in Najaf province done by Surhan *et al.*, (2018) explain that GG genotype is risk factor for HCV infection and IL-10 level is significantly increase in thalassemia patients than control and in thalassemia with HCV infection than patients with no HCV infection. Also, study done by da Silva *et al.*, (2015) they demonstrated the frequencies of alleles and genotypes related to polymorphisms in the IL-10 gene promoter showed a higher frequency of the G allele and genotype GG in the -1082 region between the infected group and the control group ($p=0.005$ and $p=0.001$, respectively), whereas the AA genotype was significantly more frequent in the control group and also observed that the genotypes GG and AG in the region -1082 were significantly more frequent among patients infected with HCV who were in advanced stages of fibrosis and cirrhosis ($p=0.042$).

In consistence with results of this study, Sheneef *et al.*, (2017) explain the regarding IL-10 -1082 G/A polymorphism genotyping, a higher frequency of GG genotype was found in chronic HCV patients compared to controls (31% versus 10%, $P < 0.05$). Sepahi *et al.*, (2014) explain the IL-10 -1082 G/A polymorphism genotyping the GG genotype was more common in patients (adjusted $p = 0.02$; OR = 4.66 [95% CI 1.31-16.35]), than control group and found a possible association between IL-10 promoter polymorphisms and HCV infection associated with higher risk or susceptibility for developing HCV infection. Sun *et al.*, (2013) demonstrate the -1082GG allele had a 43% increased risk of chronic HCV infection in combined populations (GG vs GA + AA: odds ratio (OR) = 1.433, 95% confidence interval (CI) = 1.052-1.952, $P = 0.023$), found IL-10-1082GG allele may increase the risk of chronic HCV infection in Caucasian population, and people carrying the IL-10-592A allele are more likely to clear HCV spontaneously.

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