Ruaa K. Surhan et al /J. Pharm. Sci. & Res. Vol. 10(8), 2018, 1998-2002

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

IL-10-1082A\G gene polymorphism and production in β-thalassemia major and association with HCV infection

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Abstract:

Thalassemia is the most common congenital hemolytic anemia due to partial or complete lack of synthesis of _-globin chains caused by mutations that affect the synthesis this-chains. The aim of this study is the detection of IL-10-1082A\G polymorphism and IL-10 serum level with it correlation to progression of disease.

Case-control study was performed to 60 patients with β-TM diagnosed at thalassemia-center in AL-Zahra hospital in AL-Najaf city, Iraq with group of 40 healthy individual was used as control, the patients were (26 male and 34 female) at age 3–49 from which 18 patients infected with Hepatitis C virus. Blood sample was collected from all patients and control. Blood used for DNA extracted for using SSP-PCR in detection the IL-10-1082A\G polymorphism. IL-10 level were measured by enzyme-linked immunosorbent assay test(ELISA).

The result shown that male (56.6%) more than female (42.6%), and the age range (10-19) were highest than other age range. This result explain that HCV infected patients less than non-infected thalassemia patients and the infected male more than female, the age group (10-19) was more infected with HCV. The result demonstrate that AA genotype and A allele is risk factor of severity in thalassemia patients, while GG genotype and G allele is protective factor for severity. The result explain that GG genotype is risk factor for HCV infection. This result also shown that IL-10 level is significantly increase in thalassemia patients than control, also significantly increase in IL-10 level in thalassemia with HCV infection than other patients with no HCV infection and control.

Conclusion: The polymorphism in IL-10 at position (1082A\G) has association with progression of thalassemia at AA genotype, and IL-10 consider as predictive factor for severity of thalassemia

Key words: Thalassemia, HCV, IL-10 polymorphism

INTRODUCTION:

Thalassemia is an inherited disorder of autosomal recessive gene disorder caused by impaired synthesis of one or more globin chains. Thalassemia causes varying degrees of anemia, which can range from significant to life threatening. The hematologic disorder range from asymptomatic to sever anemia that can cause morbidity and mortality [1]. It is estimated that 1.5% of the world's population are carriers of β-thalassemia with an estimated 60,000 new carries born each year [2].

Hepatitis C virus is considered high risk of thalassemia patients that acquire viral infection during multiple blood transfusions [3].

Cytokine and other regulators of the immune response may have important role in the pathogenesis of thalassemia [4]. Interleukin-10 is a vital immune-regulatory cytokine involved in both immune response and inflammation. The effects ofIL-10 upon inflammation include inhibition of pro-inflammatory cytokine production, including IL-1, IL-6, IL-12, and TNF .B-cells production .

Polymorphism in cytokine gene can influence immune response, inflammation and tissue injury and may affect the outcome of thalassemia. IL-28B(C\T) is association with pathogenicity of thalassemia disease[5]. Interleukin- 10 gene (IL-10) polymorphisms in the promoter region (known as −1082 G/A, −819 T/C, and −592 A/C) form haplotypes that are linked to different expression levels of this cytokine. In recent years, reports have demonstrated that IL10 haplotypes are associated with many aspects of different diseases and conditions, including survival and relapse in resected non-small cell lung cancer, systemic lupus erythematosus, asthma, and inhibitor development in hemophilia .

This work an attempted to study relationship between IL-10 polymorphism on the production and the effect on pathogenicity of thalassemia disease.

MATERIALS AND METHODS

The study was done at Laboratories of Bacteriology and Molecular in Biology Department, Faculty of Sciences, University of Kufa , Iraq.

Patients and Control Group

This study was carried out at the thalassemia specialized in attending to a hereditary blood disease center at Al- Zahra Teaching Hospital in AL- Najaf provenance during period from October 2016 till end of December 2016 , Participants composed of 60 patients with β-thalassemia disease they included ages ranged between(3-49)years . The patients were divided into two groups, the first group included patients infected with viral hepatitis C as well as β-thalassemia major and the second group represent β-thalassemia only, while The control group was composed of 40 randomly healthy persons (male and female). with age range between (5-47) years. Both physical and clinical examinations were done for each subject and the information was recorded in a data sheet. This study was in agreement with ethics of Al-Zahra Teaching Hospital and verbal informed consent was obtained from all participants. Blood samples and serum were collected for estimation of IL-10 serum level by sandwich ELISA, then read the results automatically by ELISA readers, using kit from Elabscience \ USA , and PCR amplification for IL10,1082A/G α polymorphism.

DNA isolation and PCR

Genomic DNA was extracted from fresh peripheral blood (3 ml in EDTA) using a commercially available kit according to the protocol of Geneius™ Micro gDNA Extraction Kit, Geneaid, USA. and then stored at -20 C till use. Single nucleotide polymorphisms (SNPs) related to the IL-10 (-1082) were determined using PCR with sequence-specific primers (PCR–SSP) in two reactions employing one common forward and two reverse primers.The reaction mix was done in 25 µl 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).The sequences of designed primers Table1 and PCR conditions for IL-0 gene are shown in Table (2).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 .

Statistical analysis

Data were translated into a computerized database structure. The database was examined for errors using range and logical data cleaning methods, and inconsistencies were remedied. An expert statistical advice was sought for. Statistical analyses were done using Graf pad prism 5 computer software .

To measure the strength of association between 2 categorical variables, such as the presence of certain genotype and disease

status the odds ratio (OR) was used The statistical significance of the measured OR is assessed by a special χ^2 formula.

Table (1) The sequences of designed specific primers

Primer	Sequences (5́-3́)	Amplicon product	Reference
IL-10-1082 G\A	F:AGCAACACTCCTCGTCGCAAC R1: CCTATCCCTACTTCCCCC R2:CCTATCCCTACTTCCCCT	179 bp	Ali and Settin.,2013 [32]

Table (2) PCR conditions for *IL-0*-1082A\G gene

Step	Temperature	Time
Initial Denaturation	94˚C	5 Minutes
Denaturation	94˚C	30 Seconds
Annealing	60˚C	60 Seconds
Extension	72˚C	60 Seconds
Final Extension	72˚C	7 Minutes

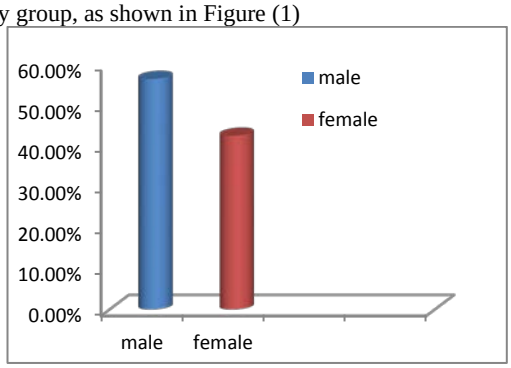
RESULTS

Demographical Distribution of Thalassemia patients

Demographic characteristics for 60 patients attending to hereditary blood disease center in AL-Najaf province by case-control study revealed the following results :

Gender Distribution

The study appear that male were 56.6% and female were 42.6% with male: female ratio 17:13 and the statically analysis revealed non-significant differences at (p>0.05) between patients and healthy group, as shown in Figure (1)



Figure(1):Distribution of the β-Thalassemia major patients according to gender

Age Distribution

The patient group were divided in to five categories according to their age ranges . The highest frequency of patient age was in 10-19 followed by 1-9,20-19 ,30-39 and 40-49 which are 31, 14, 9 ,4and 2, respectively. The results revealed the less number of older patients compare with high number in younger patients, as shown in Figure (2).

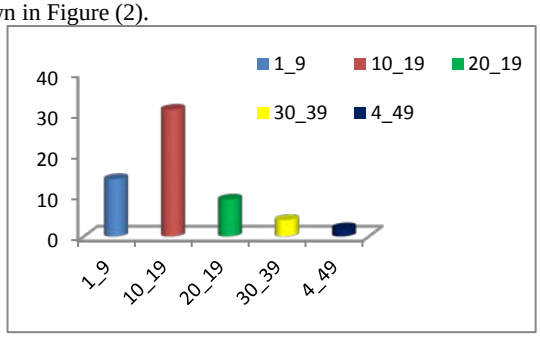


Figure (2) Distribution of β-Thalassemia patients according to age range

Hepatitis C Virus Distribution

This result demonstrate out of 60 thalassemia patients there are 22 (36.6%) sera was confirmed positive for anti- HCV- antibodies. The remaining 38 (63.3%) were seronegative. The distribution of HCV seropositive according to age group shows there was an increase in rate of infection with age of the patients. Out of 22 seropositive, 12 (20%) patients were positive among 10-19 years, followed by 5 (8.3%) was positive among 20-29 years. Out of 22 affected patient, 14(23.3%) were males and 8 (13.3%) were females, as shown in Table (3),

Table (3): Distribution of HCV infection among thalassemia patients according to age and gender

Age group (years)	HCV-infected		Non-infected HCV		Total
	Male	Female	Male	Female	
1-9	2 (3.3%)	-	2(3.3%)	10(16.6%)	14(23%)
10-19	8 (13.3%)	4(6.6%)	14(23	5(8.3%)	31(51%)
20-29	3(5%)	2(3.3%)	2(3.3%)	2(3.3%)	9(15%)
30-39	-	2(3.3%)	1(1.6%)	-	3(5%)
40-49	1 (1.6%)	-	1(1.6%)	1(1.6%)	3(5%)
Total	14(23.3%)	8(13.3%)	20(33.3%)	18(30%)	60(100%)
	22(36.6%)		38(63.3%)		60(100%)

Molecular and immunological study

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 PCR -SSP technique, at this locus there're three genotype; AA, AG and GG with band sizes of 179bp as shown in Figure (3) .

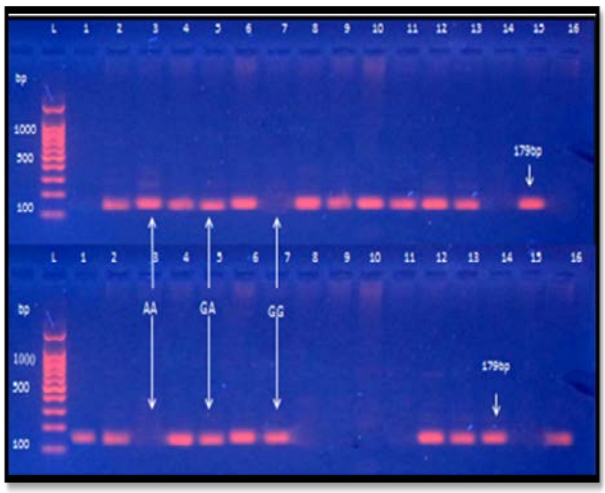


Figure (3): Ethidium bromide-stained agarose gel of PCR amplified 179bp of IL-10 gene in thalassemia patients

The genotypes frequency in thalassemia patients were as follow; AA(57.5%), AG(17.5%) & GG(25%) ; while in the healthy subjects ;AA (12.5%) ,AG (20%), GG (67.5%), Table (4)

Table (4): Genotype and Allele frequency of the IL-10-1082 A\G promoter variant among thalassemia patients and control

		Patients N=40	Control N=40	OR (95%CI)	P-value
Genotype	AA	23(57.5%)	5(12.5%)	9.4(3.06-29.2)	0.0001***
	AG	7(17.5%)	8(20%)	0.8(0.2-2.6)	No
	GG	10(25%)	27(67.5%)	0.1(0.06-0.4)	0.0001***
Allele frequency	A	53(66.5%)	18(22.5%)	6.7(3.3-13.6)	0.0001***
	G	27(33.75%)	62(77.5%)	0.1(0.07-0.2)	0.0001***

p≤ 0,05 , OR : Odds Ratio , CI: Confidence Interval

IL-10 levels among thalassemia patients that related with genotype production

		Genotype		
IL-10	M ± SE	AA	AG	GG
Patients	23.9±4.4	3.6±0.5	23.4±1.4	73.2±16.2
Control	2.9±1.5	0.2±0.1	2.3±0.4	16.4±1.001
Significant	P<0.0001***			

The study appear that mean serum level of IL-10 in patients is increase significantly than control. This result also appear significantly differences among genotype of patients and control (p <0.0001), as shown in Table (5). This result illustrate that homozygous wild type AA is give low production of IL-10, while homozygous mutant type GG give high production of IL-10, finally heterozygous AG genotype give intermediate production of IL-10.

Table (5) : IL-10 serum level in thalassemia patients and control with related to genotype

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

The result explain high frequency of GG (22.5%) than other genotype AA (10%) and AG (12.5%) in thalassemia patients infected with virus compere with non- infected patients, as shown in Table (6). The present study also 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, as shown in Figurer (4)

Table (6) correlation between genotype of IL-10 and infection with HCV in thalassemia patients

Genotype	HCV infection	Non-HCV Infection	OR (95%CI)	p-value
AA	4(10%)	19(31.6%)	0.045(0.008-0.2)	0.0001***
AG	5(12.5%)	2(3.3%)	3.8(0.6-22.8)	0.1298 no
GG	9(22.5%)	1(1.6%)	21(2.3-191.3)	0.0013**

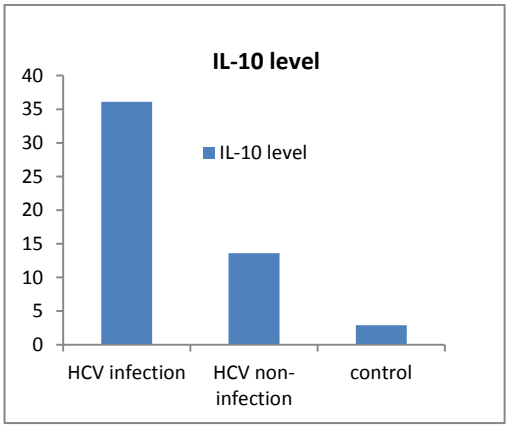


Figure (4): IL-10 serum level in thalassemia patients infected and non- infected with HCV and control

DISCUSSION

Demographical Distribution of Thalassemia patients

Gender Distribution

This study demonstrate high rate of male patients than female, this increase may be the rural and the tradition customs of rural people use to take care of male rather than female and the most patient suffering from thalassemia were rural or low income patients [6], also this results may be because male immunity is stronger than female immunity, Therefore chances of survival are longer than that of females.the result agreement with result obtained by In local study, Khaled found that the percent of male 57% were more than female 43% in Nenavha [7]. Also , Bhavsar who showed that 65% of the affected cases were males and 35% were females [8].

Age Distribution

This result show the age range (10-19) is higher than other age range , this may be due to that the patients with βTM were in the first or second decades of life, indicating lack of life expectancy [9]. This study was convenient with Muhsin and Abdul-Husin in Babylon, they found that 34.2% of patients were in the 10-20 age range [10]. A similar founded was also reported by Ali in the province of karbala, the number of age range (10-20) is 62.5% patients [11].

Hepatitis C Virus Distribution

This study show the patients suffering from thalassemia is more exposure to infection with HCV and the (10-19) age range also more exposure than other this result may be attributed to number of blood unite transfused as patients getting older due to growth, development of antibody to red blood cells and possibility of developing hypersplenism(Reference), In hand the increase chance of exposure to infected blood may be because increased frequency of admission to hospital with increase possibility for exposure to infected device or material. This result convenient with other studies like Abed in Baghdad, who founded 72.5 % male and 27.5% female [12]. Raham *et al* in Dialya and AL-Zamili *et al* in Al-Qadisiya found 20% male , 6.4% female and 53.84% male, 46.15% female respectively [13,14].

Molecular and immunological study

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

About three quarters of inter-individual variability in human IL-10 levels has been attributed to genetic variation that appear a potential role for IL-10 polymorphism in a range of human diseases like Castro-Santos *et al*, they illustrated that important associated between IL-10 polymorphism with inflammatory bowel disease and Chand-Bhayal *et al*, who revealed that patients with AA genotype more susceptible to cancer than GG genotype [15,16] . Also Esraa and Darweesh they founded that the genotypes AG were more frequently and may be contribute to the predisposition of asthma [17]. Mention other studies included IL10 polymorphism associated with thalassemia)

IL-10 levels among thalassemia patients that related with genotype production

The result of this study also explain that mean serum level of IL-10 in patients is increase significantly than control. This may be attributed to many factors such as splenectomy, iron overload and repeat exposure to foreign antigens at the time of blood transfusion or may be due to multiple blood transfusion will expose them to dangerous infection such as HIV, HBV and HCV[18]. Balouchi *et al*, they revealed that IL-10 level significantly higher than healthy and mention that immunological abnormalities have been characterized in β-TM , many of which are linked to cytokines and this imbalanced immune condition involving inflammation and immunosuppression in patients [19]. Similarly, Voskaridou *et al*, they observed that IL-10 level are increased in patients with TM and the have a role in the pathogenesis of these disorders [20].

The association of -1082 alleles G and A with a low (AA), high (GG) and intermediate (GA) IL-10 production were shown by in vivo and in vitro studies [21]. Rad *et al*, also revealed that carriers of the IL-10 -1082G allele had higher mucosal IL-10 mRNA than -1082A allele carriers The IL-10-1082 G allele has been considered to be associated with higher production of IL-10 from peripheral mononuclear cells [22]. Schaaf *et al*, 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 pneumococcal infection via induced immunosuppression and impaired bacterial clearance [23].

[24]. Sellathamby *et al*, they revealed that IL-10 are essential for both development and the effector function of CD4 regulatory T cells which are important for tolerance induction and the IL-10 polymorphism for low production were found to very significantly increase the risk of graft rejection in children with β-TM undergo bone marrow transplantation [26]. Therefore, it is possible that genotypes associated with lower production of these cytokines might contribute to increased graft rejection

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

The present study show that GG genotype more frequency in βTM that infected with HCV than AA and AG genotype and the mean of serum level increase significantly of IL-10 in patients than control. 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 [18].

Kusumoto *et al*, mention that a predominant Th1 cytokine profile responsible for recovery from an HCV infection, while predominant Th2 response that downregulates the Th1 response and develop a chronic infection (persistent HCV infection) [27]. In consistence with results of this study , Yee *et al*, explain the association of IL-10 gene promoter polymorphisms with the natural course of HCV infection [28]. Swiatek, revealed that IL-10 influence HCV infection susceptibility as well as spontaneous and treatment-induced HCV eradication [29]. In this regard Yang, founded that patients chronically infected with HCV, they produce an appropriate amounts of cytokines, IL-10, were associated with HCV clearance and even resistance to interferon therapy [30] ,Also Tsai *et al*, illustrated that IL-10 plays an important role in the pathogenesis of HCV infection [31].

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