Biochemistry Letters, 11(6) 2016, Pages: 60-70

Scientific Research & Studies Center-Faculty of Science- Zagazig University- Egypt

Biochemistry Letters

Journal home page:

The Role of Interleukin-10 Gene Polymorphism in Egyptian Patients with Chronic Hepatitis C Virus

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ARTICLEINFO

Article history:
Received
Accepted
Available online

Key words: IL-10; Polymorphism; HCV

Abstract

Background and Aim: Infection with hepatitis C virus (HCV) is the major cause of chronic liver disease worldwide, but few of patients are able to clear the virus naturally. Interleukin-10 (IL-10) is a multifunctional cytokine with anti-inflammatory properties that can suppress the immune response against HCV. Interindividual Variations in IL-10 production are genetically contributed by polymorphisms within the IL-10 promoter region. This study aimed to investigate the association of the IL-10 gene promoter (−1082 G/A) polymorphism with HCV infection susceptibility in Egyptian individuals ,also study relation between different genotypes in this promoter region and liver histopathology, in addition to studying correlation between different biochemical parameters including ALT,AST,TNF-alpha, Albumin, Total serum bilirubin, Platelets count.

Methodology: Two hundred chronically infected patients and one hundred controls were enrolled in the study. IL-10 (−1,082 G/A) genotyping was performed by amplification refractory mutation system-polymerase chain reaction (ARMS-PCR).TNF-alpha serum level was assessed by ELISA, other biochemical analysis were performed using aRoche Hitachi chemistry analyzer(USA).METAVIR scoring system was used to assess necroinflamation and fibrosis in liver biopsy.

Results: Under the dominant model for IL-10 (-1082G/A), (AA vs.GA;GG) we found a higher frequencies of A allele and GA genotype in patients group (A 53.5% : GA 45%) on the other hand; A positive correlation was found between IL-10 gene polymorphism and liver fibrosis ,also appositive correlation between ALT & AST, ALT & T.S. Billirubin, ALT & TNF-α, AST & T.S. Billirubin, AST & TNF-alpha, Billirubin& TNF- α with (P<0.001), A highly negative significant correlation between ALT & PLT, AST & PLT, TNF-alpha &Platelets (P<0.001)

Conclusions: Our findings suggest a possible association between IL-10(-1082 G/A) promoter polymorphism and HCV infection, which may confer a higher risk for developing HCV infection.

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INTRODUCTION

Hepatitis C virus (HCV) is a major cause of chronic liver disease, with more than 170 million infected individuals worldwide [1,2] and it is the most common cause of liver disease and public health problem in Egypt [3], and the highest prevalence is of HCV-4 (previously called the Egyptian genotype) which is responsible for almost 90% of infections and is considered a major cause of chronic hepatitis, liver cirrhosis, hepatocellular carcinoma and liver transplantation in the country [4].The mechanism underlying the persistence and pathogenesis is not fully understood, but probably includes virological and immunologic factors [5]. Although humoralimmunity is said to play a major role in recovery from HCV infection and B-cell is the strongest in those with persistent infection [6, 7], a strong natural killer cell mediated and Th1 cell mediated immune response seems to be a key factor in protection from HCV infection [7]. Generally, two distinct patterns of cytokine production occur .Type I responses are characterized by production of interleukin-2,tumer necrosis factor alpha (TNF alpha) and interferon gamma, which prime and maintain antigen specific cellular immunity and are important in defense against viruses [8, 9]. Type 2 responses are characterized by interleukin 4 (IL-4)and interleukin -10 (IL-10)production which promote humoral immune responses [10, 11]. An imbalance in helper T-cell type I (Th1) and type 2 (Th2) cytokine is suggested to play an important role in the pathogenesis of chronic hepatitis C [12]. Interleukin 10 (IL-10), a Th2 cytokine, is one of the many cytokines that seems to play a vital role in immune response that is generated against HCV. It shifts the Th1/Th2 balance by down regulating the Th1 responses and by suppression of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF α) and interferon gamma (IFN γ) secretion [13]. IL-10 possesses a highly polymorphic promoter with variations at -1082 G/A that have been extensively studied and implicated in altering the rates of IL-10 gene transcription. We have investigated the above-mentioned polymorphisms of the IL-10 promoter in the Egyptian population to determine if they play any role in the incidence of HCV infection in Egypt.

METHODOLOGY

Patients and controls: 200 pcr positive hepatitis C patients and 100 healthy PCR negative HCV persons were chosen from Al-Ahrar General Hospital (a local treatment center of HCV, Zagazig, Sharkia Governorate, Egypt, After approval from National committee for Prevention and Control of viral hepatitis, Ministry of Health in Egypt, To determine the association between IL-10 promoter polymorphisms at position(−1,082 G/A) and HCV infection. None of the patients had previously received any form of IFN-based therapy or hepatoprotective treatment before the study, and none of controls had a history of hepatic disease and endocrine disorders and all of them had normal liver function tests, normal liver ultrasounds and negative serological findings for viral and autoimmune liver diseases.

IL-10 (−1,082 G/A) genotyping was performed by amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) at Medical Biochemistry and Molecular Biology Department, Faculty of Medicine , Zagazig University, while other biochemical and histopathological analysis were done at Al-Ahrar hospital

Blood sampling and laboratory assays: Venous blood samples (~10 mL) were collected by trained laboratory technicians, and a complete blood count (CBC) was performed. Another portion of blood collected Invacutainer tubes containing ethylene diaminetetraacetic acid (EDTA) was stored at -80°C prior to the molecular assays.

The serum was separated to assess other biochemical measurements ,tumor necrosis factor-alpha (TNF- α) was measured by ELISA, Other biochemical Measurements were done in the laboratory including alanine amino transferase (ALT), aspartate amino transferase (AST),total serum bilirubin, serum albumin using aRoche Hitachi chemistry analyzer(USA).Liver biobsy was assessed by METAVIR scoring system.

Biochemical analysis

Biochemical analysis were performed using aRoche Hitachi chemistry analyzer (USA). Albumin was determined using Greiner diagnostic GmbH kit [14]. Quantitative determinenation of Billirubin depend on using Diamond diagnostic kit [15]. We use ELITech clinical chemistry SAS-Zone industrielle-61500 SEES FRANCE kit for determination of both alanine aminotransferas ALT & aspartate aminotransferase AST [16].

ELISA assay of serum TNF-alpha level

Serum TNF- α level was measured by acommericaly available ELISA kit with the detection limitation of 20 pg/mL (Human TNF-alpha DuoSet®, R&D Systems, MN, USA) according to the manufacturer's instructions [17].

Genotyping steps

1-DNA extraction

Genomic DNA extracted from venous blood samples preserved on EDTA of both patients and healthy control subjects using a genomic DNA extraction kit (Jena bio-science, Germany) according to the manufacturer's protocol. DNA quantification was done using an Eppendorf Bio Photometer (New York, USA). DNA was stored at 20° C.

2-Genotyping using ARMS-PCR

The amplification refractory mutation system polymerase chain reaction (ARMS-PCR) method was used for IL-10 promoter polymorphism genotyping as described by Perrey et al. [1998].In this reaction we use the allele specific for forward primers and a generic anti-sense primer, Details of primers and amplicon are shown in(Table 1). PCR amplification was performed in a 20 µl reaction volume containing 40 ng genomic DNA, 1.5 mMdNTPs, 25 mM $MgCl_2$, 1 µl of 10 pmol each primer and 0.4 units of Taq polymerase (Fermentas, Maryland, USA) in 1X Reaction Buffer with cycling conditions as follows: 95°C for 3 minutes, followed by 35 cycles at 95°C for 45 seconds, 58°C for 40 seconds, 72°C for 1 minute and finally a 7 minute extension at 72°C. To ensure PCR success, an internal control region was amplified from the human growth hormone. The amplified products were analyzed on 2% agarose gel.

Liver biopsy for HCV patients

METAVIR scoring systemwas used to assess necroinflammation and fibrosis in liver biopsy [18], Necroinflammation activity (A) was graded as A0 (absent), A1 (mild), A2 (moderate), and A3 (severe). Fibrosis stage (F) was scored as F0 (absent), F1 (portal fibrosis), F2 (portal fibrosis with few septa), F3 (septal fibrosis) and F4 (cirrhosis).

Statistical analysis

The SBSS Statistical analysis Software Version 1.0.4 (CreoStat HB Frolunda, Sweden)was used for the analysis of all data. The distribution of cytokine gene polymorphisms between HCV patients and healthy controls were compared by the chi-square test or $\chi2$. P-values smaller than 0.05 were considered significant.

RESULTS

The results show an interesting association between il-10 (-1082 G/A) polymorphism and HCV infection, as we found; a higher frequency in il-10 (-1082 A/A) genotype in patients as compared control group, where G allele is the wild prevalent allele and A is the mutant one. This explained in (Table 2).

There is a higher frequencies of A alleles at IL-10 (-1082 G/A) locus in patients group with (A 53.5%).

On the other hand frequencies of G allele at the same position in control group were significantly increase with (G 60%).

With the help of (ANOVA) we could study relation between different parameters

with each genotype in our polymorphic position.

The results show a high significant difference between three genotypes (GG,GA.AA) of patients in the mean value of (TNF-α , ALT, AST) values (p=<0.001). GG genotype has the highest mean value in TNF while AA genotype has the highest mean value for both ALT & AST. The results show a significant difference between 3 genotypes of patients in the mean value of PLTs (p=0.003).However, no significant difference between 3 genotypes in the mean value of Billirubin (p=0.33).

Liver histopathology revealed a significant higher grading of necroinflamation in patients group which are carriers of homozygotes A (A/A)genotype with (P<0.001**). In contrast no significant inflammation was detected in patients carriers of il-10 (-1082 G/G) genotype As shown in Table (4).

With studying correlation between different laboratory parameters we found; a highly positive significant correlation between; ALT & AST, ALT & T.S. Bilirubin, ALT & TNF-α, AST & T.S. Billirubin, AST & TNF- α, Billirubin& TNF- α with (P<0.001) as shown in Table (5).

DISCUSSION

There is acompelling evidence that IL-10 plays a role in HCV disease pathogenesis [19]. It was mentioned that IL-10 (aTh2 cytokine), is one of the many cytokines that seems to play a vital role in immune response that is generated against HCV. It shifts the Th1/Th2 balance by down regulating the Th1 responses and by suppression of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF α) and interferon gamma (IFN γ) secretion [13]. Due to the importance of IL-10 immune pathway, any defects in this gene can possibly make a person more susceptible to HCV infection. Therefore, the study of genetic changes, including mutations in the IL-10 gene, may help to dissect the genetic susceptibility of HCV infection [20].

The aim of this study to investigate the significance of IL-10 gene polymorphism in certain polymorphic site (-1082 G/A) and the susceptibility of HCV in Egyptian population ,and to study relation between different fibrotic markers with different genotypes in our promoter region. we use either invasive or non-invasive biomarkers. In different studies around the world, analysis of IL-10 polymorphism and its association with HCV susceptibility has produced ambiguous results [21].

In our investigation We found a highly significant association between IL-10 (-1082 G/A) gene polymorphism and HCV infection, and we found that A allele was more prevalent in HCV infected people as compared to the healthy control individuals (Table 2) & it was found that a higher frequency of il-10 (-1082 G/G)genotype in control group compared to patients one, this is in agreement with Samaneh et al. [22], Olfat et al. [23], Dogra et al. [24], Knapp et al. [25], Edwards et al. [26].

Aplausible explanation for the association of the IL-10 (-1082G/G)genotype with presistant infection is derived from experiments showing that the il-10(-1082 G)allele produces higher levels of the cytokine, which may compromise the cellular immune response to the virus [27].

However, our analysis of genotypes has shown that (heterozygous) individuals with (-1082 G/A) genotype are more susceptible to HCV infection as compared to others with(-1082G/G) (homozygous G allele). This findings suggest that cytokines may in fact compromise host immune response to the virus this was with agreement with Yoneda et al. [28] & Hanan El Bassat et al.[29], and ensure that studying the genetic changes, including mutitions in IL-10 gene may help to dissect the genetic susceptibility of HCV infection [20].

To study the relation between different fibrotic markers with different genotypes in our polymorphic site we used either invasive and non-invasive biomarkers.

For non invasive biomarkers we have measured Tumor necrosis factor alpha(TNFα) which is a cell signaling protein (adipokine) involved in systemic inflammation and plays abiovital role in the inflammatory process of hepatitis C, Tnf-α induces cell death by suppressing NF-kB activation through the action of core,NS4B,and NS5B.This mechanism is contribute to immune-mediated

liver injury in HCV infection [30]. Interestingly; our results for TNF-α levels on both cases and control groups revealed that the serum levels of TNF-α was significantly increase with il-10 gene polymorphism in our promotor region in HCV patients with AA genotype (P<0.001**) as compared to others GA & GG genotypes this is in agreement with Junseong et al.[30].

This observation are in harmony with elevated serum level of both ALT&AST in HCV patients which show highly significant increase of ALT with AA genotypes carriers (P<0.001**) as compared of GA & GG genotypes this is in agreement with Samaneh Sepahi et al. [22], Olfat G Shaker et al. [23], Hanan El Bassat et al. [29] & Marcello et al. [31], also AST show a highly significant increase with AA genotypes carriers (P<0.001**) as compared of GA & GG genotypes this was in agreement with Marcello et al. [31] while no correlation was found with AST according to Hanan El Bassat et al. [29]. In relation with platelets count we found a significant increase of platelets count in GG genotype carriers with (P=0.03*) as compared to others GA & AA genotypes. For Billirubin level there was no significant correlation between total serum bilirubin concentration &(-1082 G/A) IL-10 gene polymorphism (Table 3) this is in agreement with Hanan El Bassat et al. [29] & Dogra et al. [24].

For invasive marker (liver piobsy); For many years, liver biopsy has been considered the gold standard for the evaluation of tissue damage including fibrosis. histological assessment is based on semiquantitative scoring system(METAVIR , ISHAK score) [32]. In our study we found; a significant positive corelation between severe grading of necroinflamation & IL-10 gene polymorphism in agreement with results of Hanan El Bassat et al. [29]. We found a highly significant increase of carriers of homozygotes A and severe grading of necroinflamation with (P<0.001**)Table (4).

Conclusion ,our results are in agreement with earlier publications, our results from our study stand in contrast to previous findings on IL-10 promotor polymorphism and HCV infection in other populations. As such it is difficult to ascertain the magnitude of effect of genetic polymorphisms on disease outcome and the exact mechanisms underlying these processes remain poorly understood. Inaddition, the existence of IL-10 homologues and different IL-10 binding receptors [33] are likely to complicate the determination of levels of IL-10 expression in vitro. The interaction with these molecules could affect the correlation between IL-10 promotor polymorphisms and outcome of HCV infection. Further research on the functional implications, specifically in relation to the immune response to HCV, are clearly warranted.

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Table 1: Primers used in the study and their amplicon size.

Polymorphism/Allele location	Primer	Sequence	Product Size (bp)
Internal Control (Human Growth Hormone)	Primer 1	5'-tcacggatttctgttgtgtttc-3'	429
	Primer 2	5'-gccttcccaaccattcectta-3'	
-1082 G/A)(	Generic Primer (antisense)	5'-cagtgccaactgagaatttgg-3'	258
	Primer G (sense)	5'-ctactaaggcttctttgggag-3'	
	Primer A (sense)	5'-actactaaggcttctttgggaa-3'	

Table 2:- Genotype and allele frequencies of il-10(-1082G/A) polymorphic site among cases and controls.

	Cases		Controls		X^2	P
	N	%	N	%		
Genotype						
GG	19	19.0	33	33.0	15.38	<0.001**
GA	45	45.0	54	54.0		
AA	36	36.0	13	13.0		
Allele freq.					OR (95% CI)	
G	83	41.5	120	60.0	0.52 (0.34-0.79)	0.0012*
A	107	53.5	80	40.0		

Results are expressed as number & percentage

*P<0.001 Highly significant (HS),*P<0.05 significant(sig), *P>0.05 Non significant(NS),

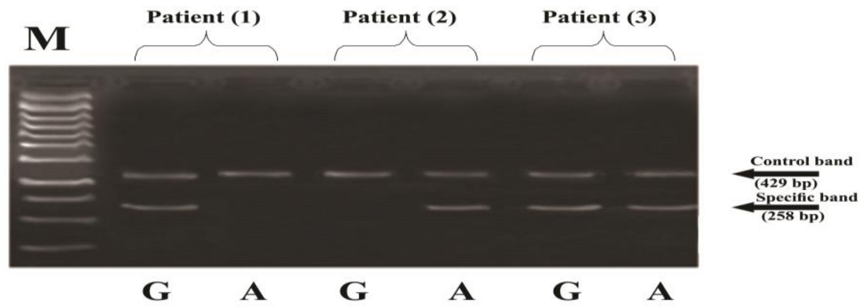


Figure 1:- Representative agarose gel electrophoresis illustrating PCR products for the il-10 promotor polymorphism in il-10(-1082 G/A)genotype locus.
M;marker
patient(1);carrier of G/G genotype.
Patient(2);carrier of A/A genotype.
Patient(3) carrier of G/A genotype.

Table 3:- Relation with patients genotype on position (-1082 G/A) of IL-10.

	GG	GA	AA	F	P
TNF					
XSD	226.150.9	261.841.9	299.644.9	17.6	<0.001**
Range	118.5-319	168.5-342.1	197.5-388.1		HS
PLT(x10^3/l)					
XSD	219.862.2	188.351.9	169.483.1	3.57	0.03*
Range	127-382	110-360	68-398		Sig.
Bil. (mg/dl)					
XSD	0.60.2	0.660.2	0.70.2	1.11	0.33
Range	0.4-1.2	0.2-1.0	0.4-1.5		NS
AST (U/L)					
XSD	29.811.9	40.120.8	59.242	7.3	<0.001**
Range	17-62	16-94	16-256		HS
ALT (U/L)					
XSD	42.219	57.726.1	8050.8	7.58	<0.001**
Range	16-89	21-152	20-259		HS

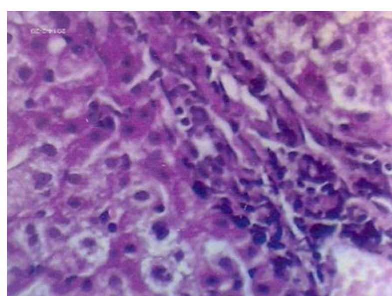
*P<0.001 Highly significant (HS),*P<0.05 significant(sig),*P>0.05 Non significant(NS),

Table 4:- Genotype with fibrosis on position (-1082 G/A) of IL-10.

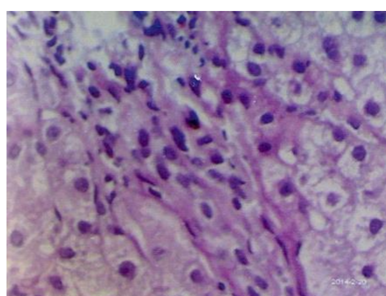
	GG N = 52		GA N = 99		AA N = 49		X^2	P
	No	%	No	%	No	%		
A								
0	33	63.5	54	54.5	13	26.5		
1	18	34.6	37	37.4	18	36.7	34.67	<0.001**
2-3	1	1.9	8	8.1	18	36.7		
F								
0	33	63.5*	54	54.5	13	26.5		
1	16	30.8	27	27.3	9	18.4	37.65	<0.001**
2-3	3	5.8	18	18.2	27	55.1*		

Necroinflammation activity (A) was graded as A0 (absent), A1 (mild), A2 (moderate), and A3 (severe). Fibrosis stage (F) was scored as F0 (absent), F1 (portal fibrosis), F2 (portal fibrosis with few septa), F3 (septal fibrosis) and F4 (cirrhosis).
*P<0.001 Highly significant (HS)

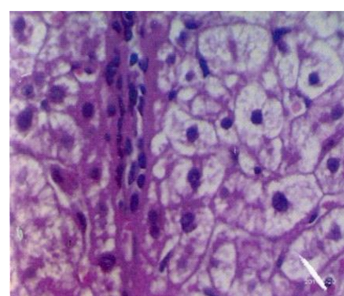
Examples for liver biopsy of different grades under microscope.



Portal tract show lymphocytic infilteration with few fibrous tissue limited to the portal area (A1F1)



Portal area shows mild lymphocytic infiltration mild fibrosis (A2F2)



Liver cells shows marked hydrophic changes (A2F3)

Table 5:- Correlation between parameters.

	R	P	Sig.
ALT & AST	0.91	<0.001	HS
ALT &Bil.	0.59	<0.001	HS
ALT & PLT	-0.48	<0.001	HS
ALT & TNF- α	0.74	<0.001	HS
AST &Bil.	0.52	<0.001	HS
AST & PLT	-0.45	<0.001	HS
AST & TNF- α	0.64	<0.001	HS
Bil. & PLT	-0.34	<0.05	Sig.
Bil. & TNF- α	0.61	<0.001	HS
PLT & TNF- α	-0.51	<0.001	HS

*P<0.001 Highly significant (HS),*P<0.05 significant(sig)

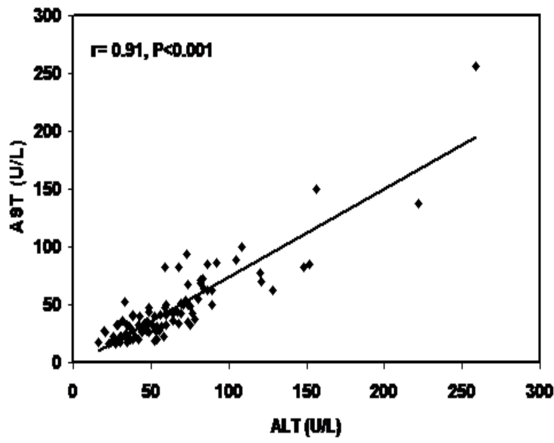


Figure 2: relation between ALT&AST.

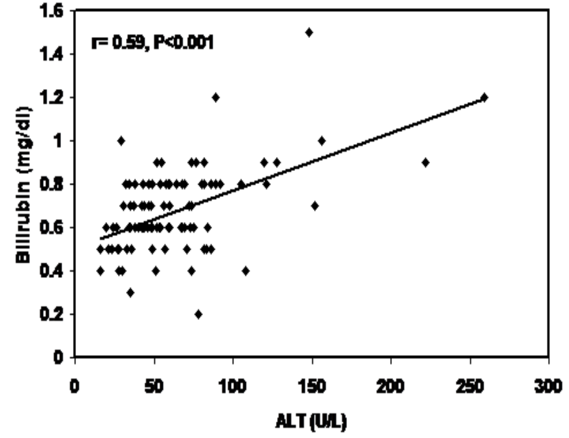


Figure 3:- relation between ALT&T.s.billirubin.

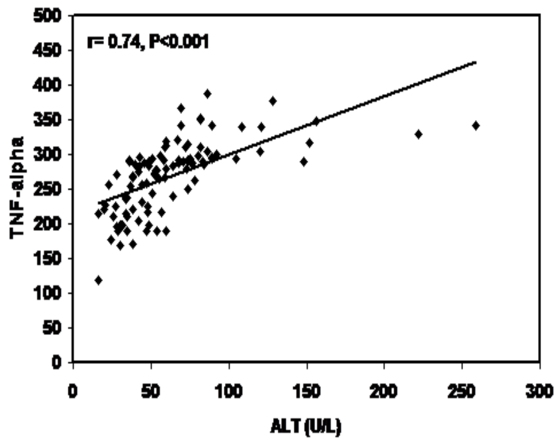


Figure 4:- relation between ALT&TNF-α

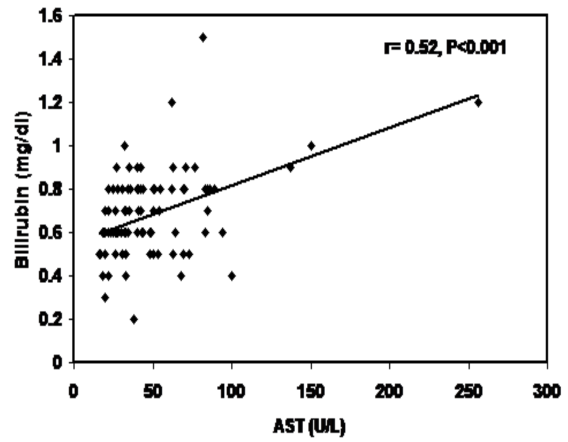


Figure 5:- relation between AST&T.s.Billirubin.

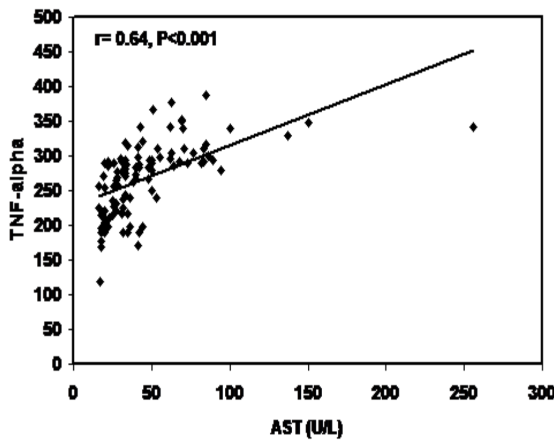


Figure 6:- relation between AST & TNF-α.

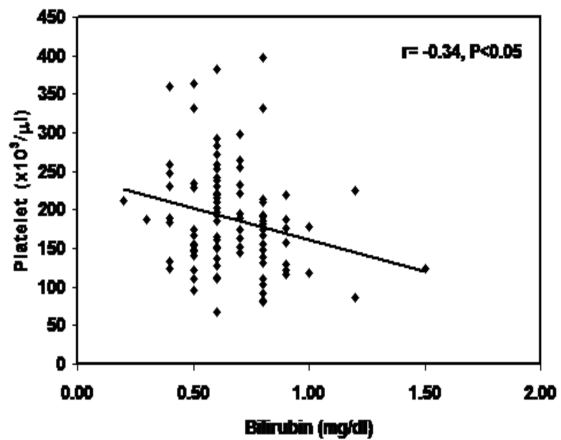


Figure 7:- relation between Platelets & T.s.Billirubin.

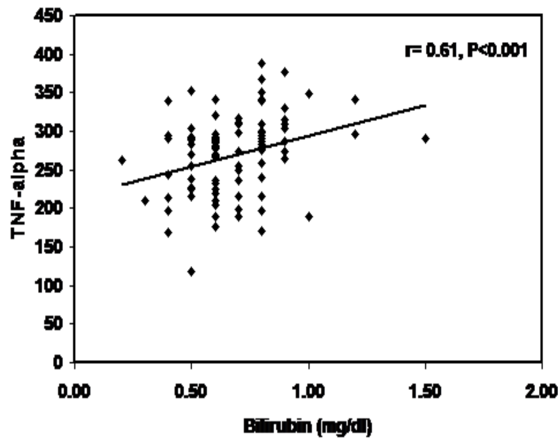


Figure 8:- relation between TNF-α & T.s. Billirubin**.**

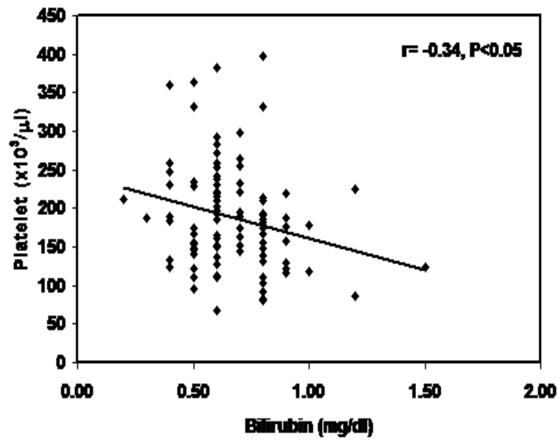


Figure 9:- relation between T.S billirubin& platelets (P<0.05).

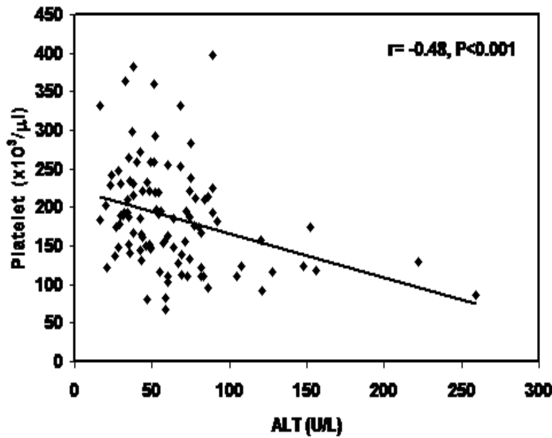


Figure 10:- relation between ALT& Platelets.

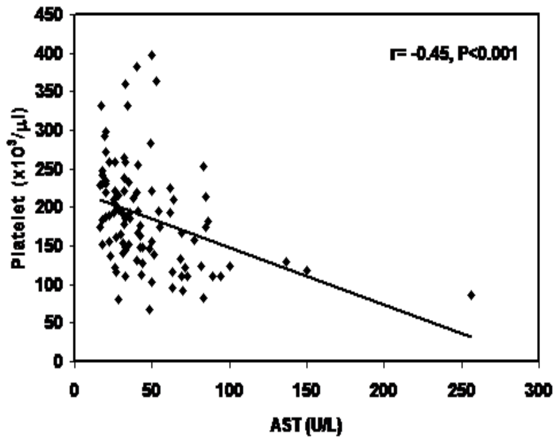


Figure 11:- relation between AST & Platelets.

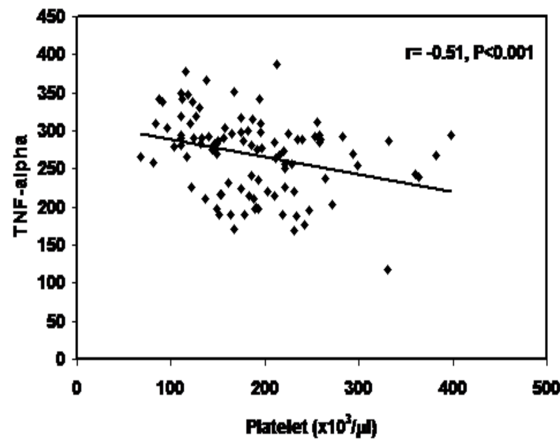


Figure 12:- relation between TNF- α & Platelets.