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Tumor Necrosis Factor-Alpha (TNF-α) Gene Expression in Chronic Hepatitis B Virus Infection

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ABSTRACT

Background: Tumor necrosis-alpha **(**TNF-α) is produced by macrophages, neutrophils, T-cells and NK-cells after stimulation. In turn, TNF-α can stimulate secretion, increase the expression of adhesion molecules as well as active neutrophils. Hence, it fulfills the role as a principal mediator of cellular immune response and inflammation, and may play an important role in non-cytopathic and cytolytic clearance of hepatitis B virus (HBV). The clearance of HBV is a complex process which may be influenced by many factors including polymorphisms in the tumor necrosis <alpha> (TNF-<alpha>) gene promoter.

Aim of the work: The study aimed to determine the TNF-α as a gene expressed in chronic hepatitis B virus infection and its role in outcome of the virus.

Patients and methods: Ninety four patients with chronic HBV infection, their age between 19 and 59 years, selected from the National Hepatology and Tropical Medicine Research Institute were included in this study, during treatment and twenty healthy individuals were included to serve as controls. All the patients and controls were subjected to the following; history, clinical examination, abdominal ultrasonography and collection of blood samples for routine laboratory investigation, and serological assay for HBsAg, HBsAb, HBeAg, HBeAb, HBV DNA (quantitative), and TNF-α promoter polymorphisms in two sites 238 and 308.

Results: The prevalence of the variant at position -308 GA was similar in all investigated groups (patients and controls). An association was found between the TNF-α promoter polymorphism at position -238 and the development of chronic HBV infection with sensitivity of 93% and specificity of 75%.

Conclusion: TNF-α-308 GA was significantly associated with clearance, showing protective antibody and persistent HBV infection. The promoter variant of TNF-α at position 238 GA, GG appears to be linked to defective viral clearance, controls had higher TNF-α-238 GG,GA, AA as compared to cases with significant difference.

Recommendations: The variation in the genes governing the levels of constitutive and inducible TNF-α might be an important factor, which might explain the variable outcome of HBV infection.

Key Words: Hepatitis B virus (HBV), Tumor necrosis factor <alpha>, polymorphism, and "SNP" single nuclueotide polymorphism at 238, and 308.

INTRODUCTION

Hepatitis B virus (HBV) infection is a major global health problem. It is estimated that over 2 billion worldwide had been infected and 400 million are chronically infected [(1)]. HBV is not directly cytopathic. The pathogenesis of HBV-related liver injury is determined by the interactions between the virus and the host immune response. [(1)] Cytokines such as tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-ð) are important for the elimination of infected hepatocytes during acute hepatitis B virus infection. [(2)] The balance between viral replication and immune responses seems to be the key to the outcome of infection. [(3, 4)] Recent data suggest that HBV may trigger weak innate immune responses following infection and that HBV rapidly develops mechanisms to repress these responses and establish a persistent infection. In chronically infected patients, intrahepatic expression of innate response genes

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is down-regulated. Furthermore, the adaptive responses are defective and are characterized by exhaustion of HBV-specific CD8+T cells. The clearance of infected cells and covalently-closed, circular DNA (cccDNA) requires a concerted action of: 1- T cell-induced lysis of infected hepatocytes followed by cell turnover to replace these cells by non-infected hepatocytes; 2- the expression of antiviral cytokines within the liver micro-environment; and 3- the production of neutralizing antibodies to prevent the infection of new cells.

The way that TNF-α inhibits HBV replication differs from other cytokines inhibitors because it targets the stability of nascent nucleocapsids. The maintenance of the cccDNA pool is thought to be critical for HBV persistence in infected hepatocytes and TNF-α mediated decline of nuclear cccDNA levels may be via preventing the formation of nucleocapsids that delivers cccDNA to the nucleus. [(5)] Type 1 IFNs likely suppress HBV messenger RNAs (mRNAs) transcription and type 11 IFNs might regulate the activity of la proteins, which may play a putative role in HBV mRNA stability. [(6)]

Nearly 11 single nucleotide polymorphisms (SNPs) in the promoter region of the TNF-α gene have been known so far including -238 G/A, -308 G/A. [(7-10)] This polymorphism can affect the TNF-α gene transcriptional activity and has different effects on serum TNF-α level. [(11-12)]

Patients and methods: Ninety four patients with chronic HBV infection, their age between 19 and 59 years, selected from the National Hepatology and Tropical Medicine Research Institute were included in this study, during treatment and twenty healthy individuals were included to serve as controls. All the patients and controls were subjected to the following; history, clinical examination, abdominal ultrasonography and collection of blood samples for routine laboratory investigation, and serological assay for HBsAg, HBsAb, HBeAg, HBeAb, HBV DNA (quantitative), and TNF-α promoter polymorphisms in two sites 238 and 308.

(308) DNA Purification from blood: This protocol is for purification of (genomic and viral) DNA from whole blood using a microcentrifuge. For total DNA purification using a vacuum manifold.

Important points before starting: 1- All out at room temperature (15-25 °C). 2- Use carrier DNA if the sample contains < 10,000 genome equivalents. 3- 200 ul of whole blood yields 3-12 ug of DNA. Preparation of buffy coat is recommended if a higher yield °C is required.

Things to do before starting: 1- Equilibrate samples to temperature (15-25 °C). 2- Heat a water bath or heating block to 56 °C for use in step 4. 3- Equilibrate buffer AE or distilled water to room temperature for elution in step 11. 4- Ensure that buffer AW1, buffer AW2, and a QIAGEN protease have been prepared according to the instructions. If a precipitate has formed in buffer AL, dissolve by incubating at 56 °C.

Procedure:

1- Pipit 20 ul QIAGEN protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube.

2- Add 200 ul sample to the microcentrifuge tube. Use up to 200 ul whole blood in 200 PBS. If the sample volume is less than 200 ul, add the appropriate volume of PBS. QIAamp Mini spin columns copurify RNA and DNA when both are present in the sample. RNA may inhibit some downstream enzymatic reaction, but not PCR. If RNA-free genomic DNA is required, 4 ul of an RNase A stock solution (100 mg/ml) should be added to the sample before addition of buffer AL.

Note: It is possible to add QIAGEN protease (or proteinase K) to samples that have already been dispensed into microcentrifuge tubes. In this case, it is important to ensure proper mixing after adding the enzyme.

3- Add 200 ul buffer AL to the sample. Mix by pulse-vortexing for 15 s.

To ensure efficient lysis, it is essential that sample and buffer AL are mixed thoroughly to yield a homogeneous solution. If the sample volume is larger than 200 ul, increase the amount of QIAGEN protease (or proteinase K) and buffer AL proportionally, for example, a 400 ul sample will require 40 ul QIAGEN protease (or proteinase K) and 400 ul buffer AL. If sample volumes larger than 400 ul are required, use of QIAamp DNA blood Midi or

Maxi Kits is recommended; these can process up to 2 ml or up to 10 ml of sample, respectively.
Note: Do not add QIAGEN protease or proteinase K directly to buffer AL.
4- Incubate at 56 °C for 10 min. DNA yield reaches a maximum after lysis for 10 min at 56 °C longer incubation times have no effect on yield or quality of the purified DNA.
5- Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
6- Add 200 ul ethanol (96-100%) to the sample and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid. If the sample volume is greater than 200 ul, increase the amount of ethanol proportionally; for example, a 400 ul sample will require 400 ul of ethanol.
7- Carefully apply the mixture from step 6 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 × g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate. Close each spin column to avoid aerosol formation during centrifugation. Centrifugation is performed at 6000 × g (8000 rpm) to reduce noise. Centrifugation at full speed will not affect the yield or purity of the DNA. If the lysate has not completely passed through the column after centrifugation, centrifuge again at higher speed until the QIAamp Mini spin column is empty.
Note: When preparing DNA from buffy coat, centrifugation at full speed is recommended to avoid clogging.
8- Carefully open the QIAamp Mini spin column and add 500 ul buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 × g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate. It is not necessary to increase the volume of buffer AW1 if the original sample volume is larger than 200 ul. Flow-through contains buffer AL or buffer AW1 and is therefore not compatible with bleach.
9- Carefully open the QIAamp Mini spin column and add 500 ul buffer AW2 without wetting the rim. Close the cap centrifuge at full speed (20,000 × g; 14,000 rpm) for 3 min.
10- Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible buffer AW2 carryover.
11- Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 ul buffer AE or distilled water. Incubate at room temperature (15-25 °C) for 1 min, and then centrifuge at 6000 × g (8000 rpm) for 1 min. Incubating the QIAamp Mini spin column loaded with buffer AE or water for 5 min at room temperature before centrifugation generally increases DNA yield.

A second elution step with a further 200 ul buffer AE will increase yields by up to 15%. Volumes of more than 200 ul should not be eluted into a 1.5 ml microcentrifuge tube because the spin column will come into contact with the eluate, leading to possible aerosol formation during centrifugation.

Elution with volumes of less than 200 ul increases the final DNA concentration in the eluate significantly, but slightly reduces the overall DNA yield. For samples containing less than 1ug of DNA, elution in 50 ul buffer AE or water is recommended. Eluting with 2 × 100 ul instead of 1 × 200 ul does not increase elution efficiency. For long-term storage of DNA, eluting in buffer AE and storing at -20 °C is recommended, since DNA stored in water is subjected to acid hydrolysis. A 200 ul sample of whole human blood (approximately 5×10^6 leukocytes/ml) typically yields 6 ug of DNA IN 200 ul water (30 ng/ul) with an A_{260}/A_{280} ratio of 1.7-1.9. For more information about elution and how to determine DNA yield, purity, and length. [13]

Blood Sampling and 238 DNA Extraction:

Peripheral blood samples were collected in EDTA-tubes.

Genomic DNA was extracted from all blood samples by Gene JET™ Genomic DNA Purification Kit (#K0722) according to the manufacturer's instructions and the purified

DNA was either immediately used in downstream applications or stored at -20 °C.

Restriction Fragment Length Polymorphism (RFLP)

All DNA samples extracted were subjected to PCR to amplify 238 region of the TNF-α gene promoter.

-238 G/A polymorphism, the forward primer 5'-AGAAGACCCCCCTCGGAACC-3' and the reverse primer 5'-ATCTGGAGGAAGCGGTAGTG-3'were used. Each 25-mL PCR reaction contained 2.5 uL of 10X PCR buffer, 1.5 mMol of MgCl2, 10 pmol of each primer, 0.2 mM of the dNTPs, 15 uL of deionized water, 1 μL of Taq DNA polymerase, and 40-50 ng of genomic DNA as a template. The mixture was denatured at 95 ºC for 5 minutes and underwent 35 Cycles in a thermocycler PCR system under the following conditions: denaturation at 95ºC for 1 minute, annealing at 60 ºC for 45 seconds, extension at 72ºC for 1 minute, and a final extension for 10 minutes at 72 ºC. The amplified fragments were detected on 2% agarose gel (Invitrogen, Carlsbad, California, USA). All PCR reactions were performed and visualized as previously described. PCR products of all subjects were restricted with MspI enzyme (#ER0542) for investigating -238 G/A polymorphism. Both restriction reactions were performed in a 30 μl scale

reaction as described before. [14] Restriction reactions were run side by side with an equivalent amount of undigested PCR reaction mixture in 5 % agarose gels for 50 min in TBE buffer and the gels were stained and photographed.

Statistical analysis: Analysis of data of all patients was done by IBM computer using SPSS (Statistical Program for Social Science version 16) as follows: Description of quantitative variables as mean, SD and range. Description of qualitative variables as number and percentage. Chi-square test was used to compare qualitative variables between groups. Unpaired t-test was used to compare two independent groups as regard quantitative variable. Mann Whitney Willcoxon U test was used instead of t-test in non parametric data (SD>50% mean). ROC Curve (receiver operator characteristic curve) was used to find out the best cut off value, and validity of certain variable. [15]

Ethical consideration: Informed consent was obtained from each patient at the time of drawing blood samples. The Research Ethical Committee of the General Organization for Teaching Hospitals and Institutes approved the study protocol.

RESULTS

This study included 94 patients with CHBV infection and 20 healthy volunteers.

We found that the controls had higher levels of TNF-α-238 as compared to chronic HBV patients with significant difference by using Mann Whitney test ($p<0.001$) table (1).

Table (1): Also shows no statistically significant difference between CHBV patients and controls as regard TNF-α-238 GG,GA, AA different alleles by using Chi-Square test ($p>0.5$).

Graph (1): Illustrates that serum TNF-α levels was higher among chronic HBV patients as compared to controls.

Table (2): Shows no statistically significant association between TNF-α-238 as regarding the PCR (Polymerase chain reaction), results (response to treatment after more than 2 years).

Note: The samples of CHBV patients included in the study received different antiviral treatment: eg. Lamidine, Tenaviron, IFN, Viridine, Tecviridine+ Lamidine and Baraclude.

Table (3): Shows that 100 % of chronic HBV patients as regarding TNF-α-308 are GA alleles had a response to treatment (PCR results) below detection limits (BDL) by 78 %.

Table (4): Shows no statistically significant difference of TNF-α-238 in chronic HBV patients as regarding the response to treatment by PCR results by using Mann Whitney test.

Table (5): Shows no statistically significant correlation between TNF-α-238 versus PCR results or AFP (alpha foeto protein) by using Spearman correlation test.

Graph (2): Illustrates that the sensitivity of TNF-α-238 in chronic HBV patients was 93 %, and that the specificity was 73 % by ROC Curve.

Table (6): Shows the high validity of TNF-α-238 in diagnosis of chronic HBV infection.

Graph (3): Illustrates the same results of graph (2), and table (6).

DISCUSSION

The clearance of hepatitis B virus (HBV) is a complex process which may be influenced by many factors including polymorphisms in the tumor necrosis factor <alpha> (TNF-<alpha>) gene promoter. [16] The TNF-α gene is located in the class III region of the major histocompatibility complex (MHC) on chromosome 6. The amount of cytokine production seems to be affected by the polymorphisms in the regulatory region. Therefore, there might be relationships between these single nucleotide polymorphisms (SNPs) and cytokine mediated inflammation, which may affect the outcome of the disease. [17-20] In addition to host factors, several viral factors have been reported to affect the innate or adaptive immune responses against HBV infection. The hepatitis e antigen (HBeAg) is a viral immunomodulatory protein that, via deletion or anergy, inhibits the HBV core (HBcAg) HBeAg cross-reactive T-cell response. [21] The soluble hepatitis B surface antigen (HBsAg) significantly exhausts HBsAg T-cell responses. [22] HBV polymerase blocks pattern-recognition receptor. [23] Furthermore, it is likely that sequence diversity between different HBV genotypes or different HBV strains may influence the existence of particular epitopes, thus resulting in different immune response profiles. [24-26]

The genetic background of the host and viral factors are believed to contribute to the different outcomes of HBV infection. Genetic polymorphisms of several host factors have been implicated in the susceptibility to chronic HBV infection, including tumor necrosis factor-alpha (TNF-α) promoter. [27-29]

In the present study, we found that high validity of TNF-α-238 in diagnosis of chronic HBV infection with sensitivity of 93 % and specificity of 75 %, and there's no statistically significant difference between the patients and controls as regard TNF-α-238 alleles, and also that controls had higher TNF-α-238 as compared to cases with significant difference. Whitten et al. [30], Foster et al. [31], observed that transactivating /inhibiting factors of HBV might show different interaction with the TNF 238.2 allele and cause decreased transcription of the TNF gene. Failure to secrete adequate amounts of TNF-α could possibly prevent viral clearance and lead to chronic infection; these results are in agreements with our results. Although one study has claimed that TNF-α-238 A allele may increase the risk of chronic HBV infection in European populations however, no significant associations were found in Asian populations in all genetic models. [32]

In our study, we observed that 100 % of patients are GA alleles as regarding TNF-α-308. Tayebi et al. [33] postulated that TNF-α-308 allele promoter polymorphism has been known to be a potential prognostic factor in patient with chronic HBV infection, and also they added that there is a positive relation between TNF-α-G-A-308 promoter polymorphisms and resolution of chronic HBV infection. The significant role of tumour necrosis factor (TNF-α) in inflammation process has been attracted a great attention in both the regulation of the TNF-α gene and the possibility of TNF-α variants production. Polymorphisms in particular, at position-308, are reportedly capable of altering TNF-α expression. Also Tayebi et al. [33] supposed that binding of cellular factors to TNF-α promoter might be influenced by this polymorphism and affect gene expression and disease outcome all these finding were correlated with our results.

There are 3 different kinds of results about the relationship between TNF-α-308 gene promoter polymorphism and prognosis in patients with chronic HBV infection: the first group of studies is about to say "there is no association". One study from Japan showed that allelic distributions of both gene promoters (including TNF-α and IL-10) were not significantly different between HBV carriers and healthy volunteers [34]. Another study from Italy demonstrated that TNF-α gene promoter polymorphisms do not appear to be determinant of HBV seroclearance. [19] Despite the fact of high prevalence of TNF-α gene promoter polymorphism in -308 locus in Iran. It has no association with development of chronic HBV infection. [35]

The second group of studies are about to say "there is positive association between TNF-α-308 gene promoter polymorphisms are associated with either "unfavorable prognosis of chronic HBV infection" or high risk of persistent HBV infection. Korean patients with TNF-α-308

gene promoter polymorphisms had higher risks of persistent HBV infection. [28] The genotype -308 G/G and haplotype TGGG are associated with an unfavorable prognosis in patients with chronic HBV infection. [19] In Chinese people, frequency of haplotype GGCCT (-238, -308) in patients with chronic HBV infection was significantly lower than that in spontaneously recovered group. [36]

The third group of studies are about to say "there is a positive association between TNF-α-308 gene promoter polymorphisms and resolution of HBV infection". It has been shown that TNF-α-308 G/A or A/A promoter polymorphisms are associated with HBV clearance [18]. A meta-analysis about TNF-α-308 gene promoter polymorphisms has claimed that TNF-α-308 A allele may have a protective effect on the prognosis of chronic HBV infection in Mongoloid populations. [37]

In the present work we observed that serum TNF-α levels were higher among chronic HBV patients as compared to controls. Mohamadkhani et al. [38] postulated that circulating TNF-α level increases during HBV infection. Increased hepatic level of TNF-α is associated with suppression of HBV replication in transgenic mice which expresses HBV in the liver. [39]

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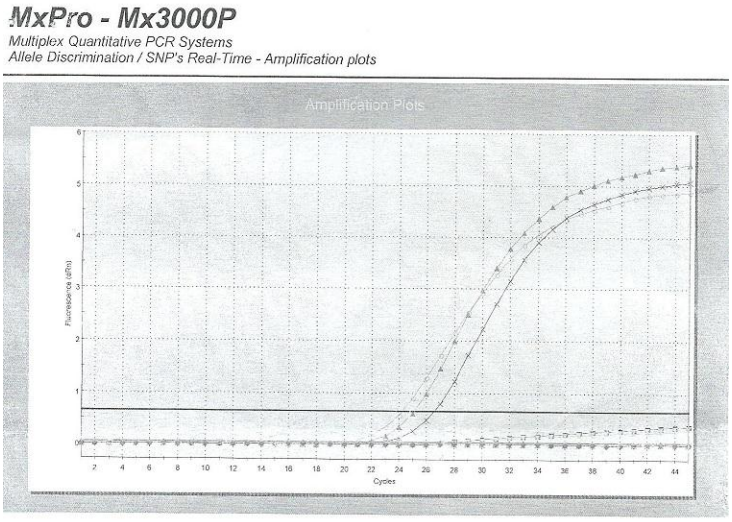


Fig (1): Illustrates the TNF-α-308 SNPs Real-Time-Amplification Plots quantitative PCR systems-allele discrimination.

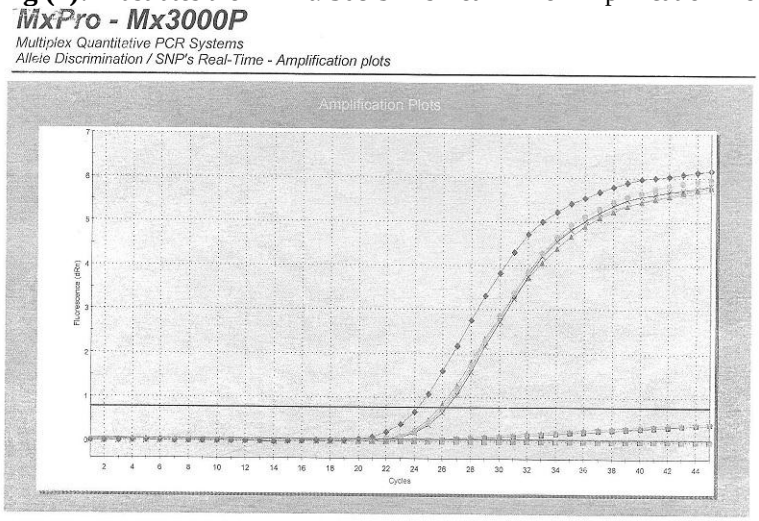


Fig (2): Illustrates the TNF-α-308 SNPs Real-Time-Amplification Plots quantitative PCR systems-allele discrimination

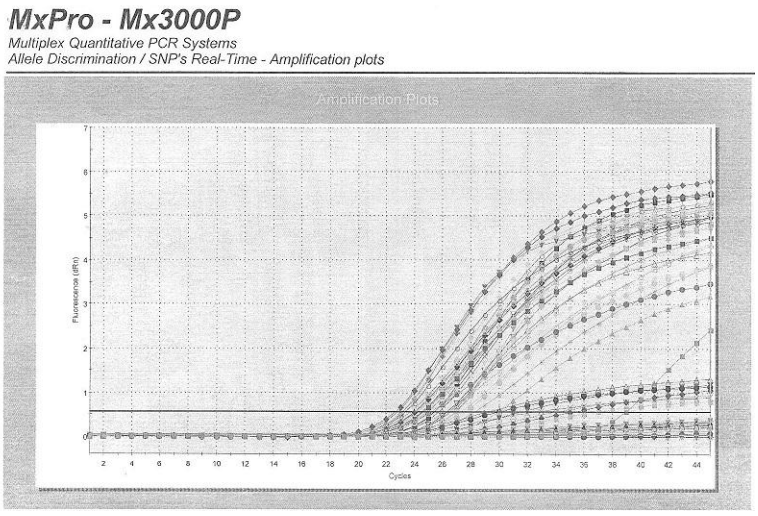


Fig (3): Illustrates the TNF-α-308 SNPs Real-Time-Amplification Plots quantitative PCR systems-allele discrimination.

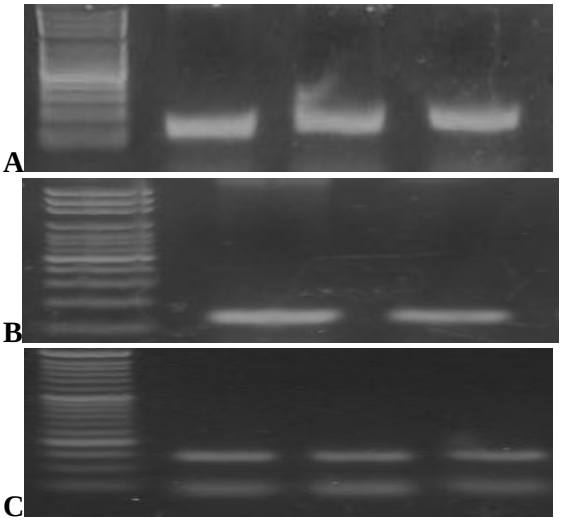


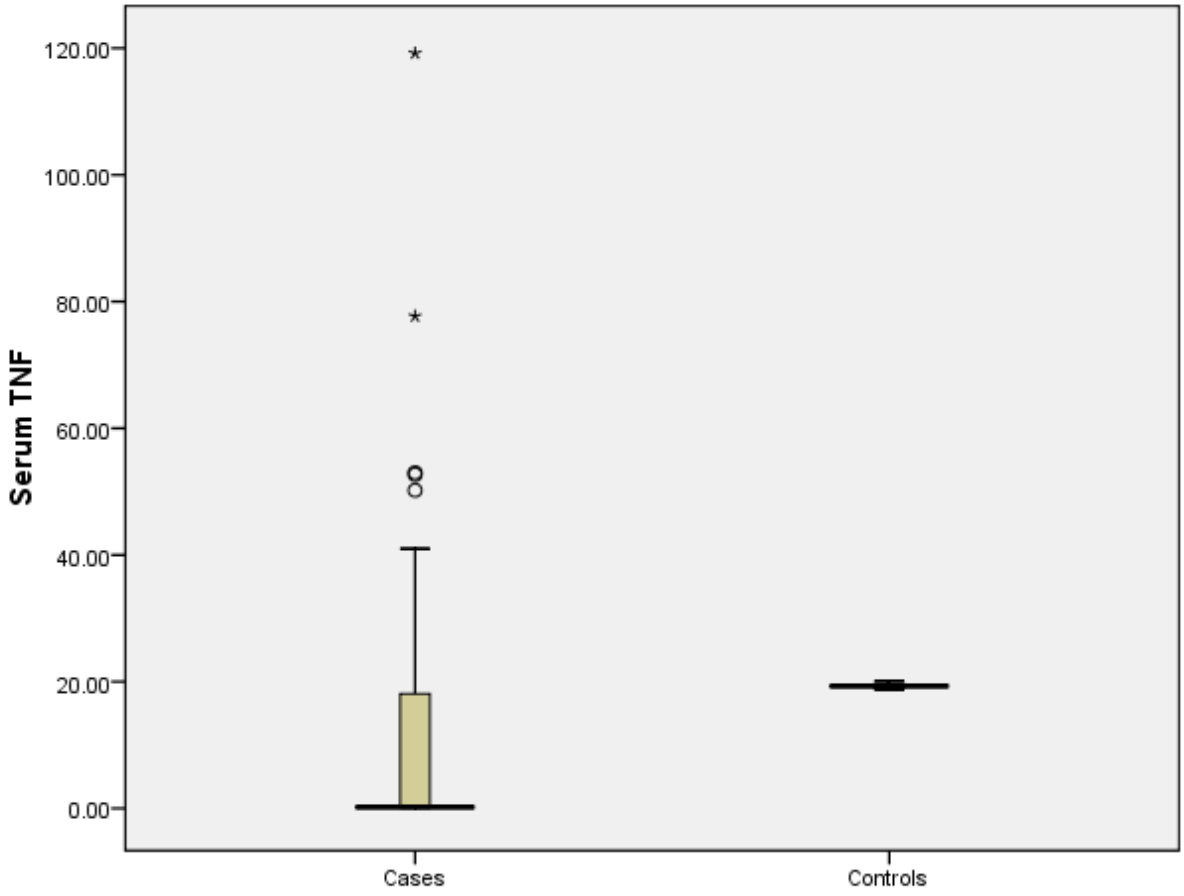
Fig (4): Illustrates the TNF-α-238-SNP, s PCR products an agarose gel electrophoresis.

An agarose gel electrophoresis show PCR products of TNF-alpha gene polymorphism (238).
GG genotyping (A) with 154bp products.
AA genotyping (B) with 132bp products.
GA genotyping(C) with 154&132 bp product.

Table (1) Comparison between cases and controls as regard TNF-α-238 different alleles.

TNF238	**Cases** N=94	**Controls** N=15	X^2	**P**
AA	4(4.3%)	0	1.14	0.56 NS*
GA	28(29.8%)	6(40%)		
GG	62(66%)	9(60%)		
Serum (TNF) mean±SD Median (IQR)	 12.6±7 0.2(0.1-18.7)	 19.6±8 19.3(18.9-19.7)	**3#**	**0.001 S****

#**Mann Whitney test** ***Non significant** **** Significant**



Graph (1): Illustrates the comparison between CHBV cases and controls as regard serum TNF-α.

Table (2) Comparison between TNF-α-238 versus response to treatment by PCR results BDL among CHBV cases.

TNF238	**Response** Positive	BDL	**X^2**	**P**
AA	0(0%)	4(5.1%)	4.2	0.12 NS*****
GA	8(50%)	20(25.6%)		
GG	8(50%)	54(69.2%)		

***Non significant**

Table (3)
Comparison between TNF-α-308 versus response to tretment among CHBV cases.

TNF308	**Response** Positive	BDL	**X^2**	**P**
GA	16(100%)	78(100%)	-	-

Table (4) Comparison between serum TNF-α-238 versus response to treatment among cases.

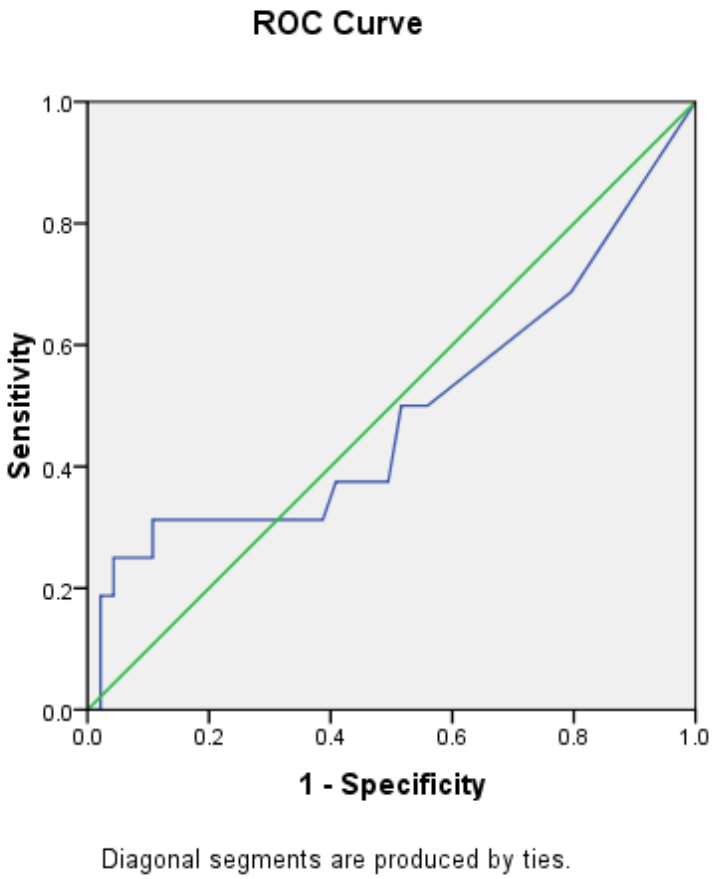
TNF238	**Response**		**Z**	**P**
	Positive	BDL		
Mean±SD	16.5±11	11.9±7	0.86	0.39 NS*

***Non significant**

Table (5) Correlation between TNF-α-238 versus PCR and AFP among CHBV cases.

TNF238	**Serum TNF**	
	r	**P**
PCR	-0.09	0.36 NS*
AFP	-0.06	0.56 NS*

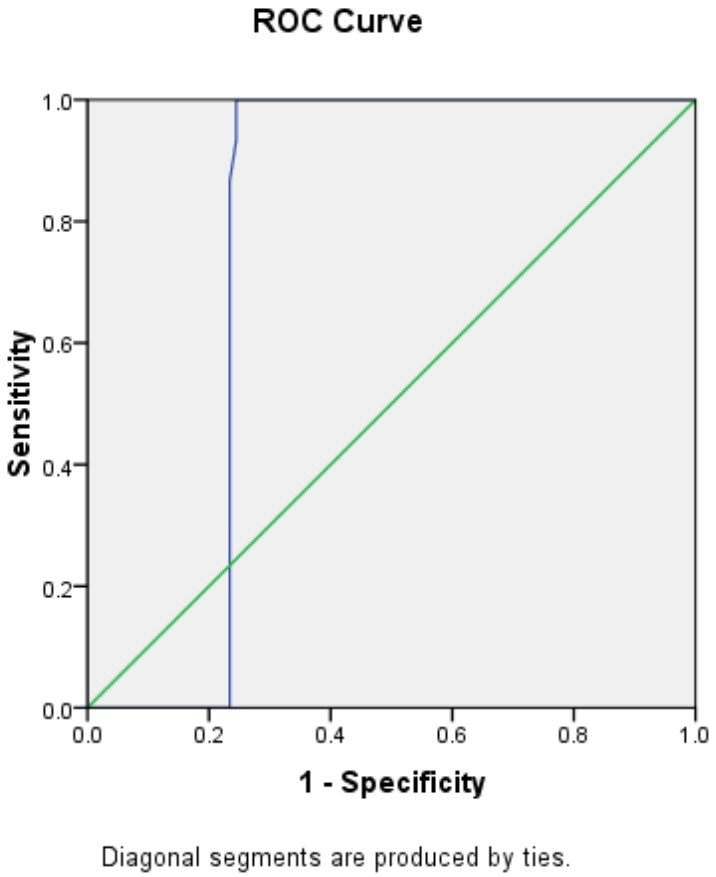
***Non significant**



Graph (2): Illustrates the sensitivity and specificity of TNF-α-238 in CHBV patients.

Table (6) Validity of TNF-α-238 in diagnosis of chronic HBV infection.

Variables	**%**
Best cut off	18.7
Area under the curve (AUC)	0.76
Sensitivity	93%
Specificity	75%
PPV	80%
NPV	95%



Graph (3): Illustrates the sensitivity and specificity of TNF-α-238 in CHBV patients.