

Expression of toll like 3 in chronic hepatitis c virus infection, Short title: Expression of toll like 3 in Hepatitis

Dalia El Sayed Sherief, Amaa. Emubark Hassan, Eman Mohamed Habeeb

Clinical pathology department, Faculty of Medicine, Kafr El-Sheikh University, Kafr El-Sheikh, Egypt,
Corresponding author: D.E. Sherief¹; Elgeish St., Kafr El-Sheikh University hospitals, Kafr El-Sheikh, EgyptJust2008@windowslive.com

Tel.: 20/010980330898

Abstract: Introduction: Of the innate immune response Toll-Like Receptors (TLRs), are important components that recognize pathogen-associated molecular patterns (PAMPs) and trigger immune responses. TLRs could contribute to cirrhosis and HCC as TLR3 signaling negatively regulates liver regeneration via stimulating NK cells to produce IFN. **Aim of the work:** To detect level of TLR3 gene expression in patients with hepatitis c, its correlation with INF- α and other parameter. **Patients and methods:** This study was done on sixty patients with hepatitis c from Kafr Elsheikh university hospital and sixty healthy control. **Results:** There was highly significant increase of serum INF alpha levels in patients when compared with control group, as regard TLR 3 gene expression by comparing patients against control there was highly significant difference. There was significant positive correlation between TLR 3 gene expression and INF alpha level in patients. There was no significant correlation between TLR 3 gene expression and (Age, ALT, AST, ALB, Total Bilirubin, HB, PLT, TLC). **Conclusion:** The results of this study suggested that Toll-like receptor3 has a great role in host response against HCV infection. There is significant positive correlation between TLR 3 gene expression and INF alpha level in patients. The down regulation of TLR3 expression by HCV may contribute to the decrease of IFN- α in HCV patients.

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Key Words: Hepatitis c, Interferon α , TLR3.**Introduction:**

In Egypt hepatitis c is the most common cause of chronic liver disease (CLD). The inflammatory and wound-healing responses that is triggered in response to chronic liver injury in the long run promote the development of hepatic fibrosis and HCC (*Mohamoud, et al., 2013*).

The innate immune system can responds to HCV by activation of TLRs that lead to a series of signaling events resulting in the production of IFN- α , IFN- β , IFN- γ , and other TLR-induced inflammatory cytokines. The expression of TLR3 is dominant on parenchymal and nonparenchymal cells in the liver as well as in many types of immune cells including macrophages, dendritic cells, NK cells, NKT cells (*Yin, et al., 2010*).

There is a growing evidence indicating that TLR3 plays a role in cirrhosis pathogenesis and hepatocarcinogenesis. It has been documented that dsRNA activates TLR3 which subsequently results in NK cell accumulation and activation leading to inflammation of the liver. Such process could contribute to cirrhosis and HCC if left untreated (*Al-Anazi, et al., 2017*).

Aim Of The Work:

The aim of the work is to determine the relative

level of interferon α and TLR3 expression in patients with chronic HCV infection.

Patients, Materials And Methods:

This study included 60 patients together with 60 healthy control referred to Tropical Medicine Department, Kafr Elsheikh University Hospital, informed consents was obtained from all the patients and controls. The diagnosis was established on the basis of history, clinical, laboratory, abdominal ultrasound. CBC, liver function tests, HBS antigen & anti-HCV antibody, interferon α and TLR3 expression were also done.

- **Inclusion Criteria:** Adult patients (>18 years), both sexes (males or females) and patients with CHCV infection.

- **Exclusion Criteria:** Patients who had received antiviral treatment, patients with HIV or HBV co-infections and patients with HCC.

- **Laboratory investigations:** Blood samples were collected from the patients at the time of diagnosis, before any kind of treatment (surgery, radiation, or chemotherapy) as well as from the control group. Each blood sample was divided into three portions as follows:

- First portion was collected into Na citrate-containing tube, and used for estimation of

prothrombin time (PT) immediately on automated blood coagulation analyser (stago).

- The second portion was collected into EDTA containing tube for CBC estimation using Coulter Counter T890 (Coulter LH 750 analyzer, Berlin, Germany).

- The third portion was put in a plain tube, left to clot then centrifuged at 1600 rpm for 20minutes and serum was separated and used for estimation of:

- Liver and kidney function tests were done on Hitachi 911 auto-analyzer (Roche-Hitachi, Japan).

- Detection of HBS antigen & anti-HCV antibody by enzyme-linked immunosorbent assay (ELISA).

- The presence of HCV RNA by reverse transcription- polymerase chain reaction (RT-PCR).

- Serum Alpha fetoprotein (AFP) was detected by COBAS e411 chemiluminescence auto analyzer using Roche reagents (Roche Diagnostics GmbH, D-68289 Mannheim, Germany).

- Estimation of interferon α by ELISA kit (Verikine Human IFN Alpha supplied from pbl assay Science catalog NO4110) with extended range 156-5000 pg/ml. This kit quatitates the human interferon alpha in the media using a sandwich immunoassay. The kit is based on ELISA with anti detection antibody conjugated to horseradish peroxidase (HRP). Tetra methyl-benzidine (TMB) is substrate. The assay is based on international reference standard for human interferon alpha provided by national institute of health.

- Determination of relative level of TLR3expression by real time PCR. Gene Expression Analysis Real time PCR amplification was done by Rotor - Gene Q Real Time PCR instruments from QIAGEN USA. Using real-time PCR kit (Lot Number 1091352 Rev. APPLIED BIOSYSTEMS. 03/2017). Total RNA was extracted from peripheral venous blood of each individual using RNA extraction and purification kit (QIAamp RNA Blood Mini Kit, Qiagen, Germany) in fully automated on the QIAcube (Germany).

- Isolated total RNA was quantified photo spectrometrically at 260 nm. Purity of total RNA was assessed by the 260/280 nm ratio which was between

1.8 and 2.1. The RNA integrity was tested on the Nano drop ND-1000 spectrophotometer (Germany) which is a full spectrum (220 -750nm) spectrophotometer that measures 1 μ l samples with high accuracy and reproducibility. Immediately after blood drawing with the RNEASY Blood RNA system (Quiagen, Dusseldorf, Germany). The mRNA was transcribed into cDNA, using the Omniscript reverse transcription kit (Quiagen, Dusseldorf, Germany).

- QIAGEN's real-time PCR cycler (Rotor-Gene Q, USA) was used to determine the cortex DNA copy number. PCR reactions were set up in 25 μ L reaction mixtures containing 12.5 μ L 1 \times SYBR[®] Premix ExTaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 μ L 0.2 μ M sense primer, 0.5 μ L 0.2 μ M antisense primer, 6.5 μ L distilled water, and 5 μ L of cDNA template. The reaction program was allocated to 3 steps. First step was at 95.0 $^{\circ}$ C for 3 min. Second step consisted of 40 cycles in which each cycle divided to 3 steps: (a) at 95.0 $^{\circ}$ C for 15 sec; (b) at 55.0 $^{\circ}$ C for 30 sec; and (c) at 72.0 $^{\circ}$ C for 30 sec. The third step consisted of 71 cycles which started at 60.0 $^{\circ}$ C and then increased about 0.5 $^{\circ}$ C every 10 sec up to 95.0 $^{\circ}$ C. The fluorescence products were differentiated by ABI sequencer 377(Applied Biosystems). The sequences of specific primer of the TLR 3 are forward primer TGGTTGGGCCACCTAGAAGTA and reverse primer: TCTCCATTCTGGCCTGTG.

Results:

The current study included 120 subjects 60 patients 32(53.3%) males & 28 (46.7%) females, their ages ranged from 34 to 67 years and 60 healthy subjects as a control group. The results and data were collected and analyzed in tables 1-6 and figures 1-3.

As regard serum INF alpha there was significant increase in patients (mean=39.90 \pm 67.83) (P<0.05) as compared to controls (mean= 10.20 \pm 1.20). There was significant difference as regard TLR 3 gene expression by comparing patients against controls {Table (5) and Fig. (2)}. There was significant positive correlation between TL 3 gene expression and INF alpha level in patients, while there was no significant correlation between TL 3 gene expression and (Age, ALT, AST, ALB, Total Bilirubin, HB, PLT and TLC).

Table (1): Age and sex distribution of the studied groups

		Patients	Control	P-value
Age	Mean \pm SD	49.37 \pm 8.4	49.70 \pm 9.26	0.304
sex	Males	No.	32	24
		%	53.3%	40.0%
	Females	NO.	28	36
		%	46.7%	60.0%
	Total		60	60

Table (2): Comparison of complete blood picture between the studied groups:

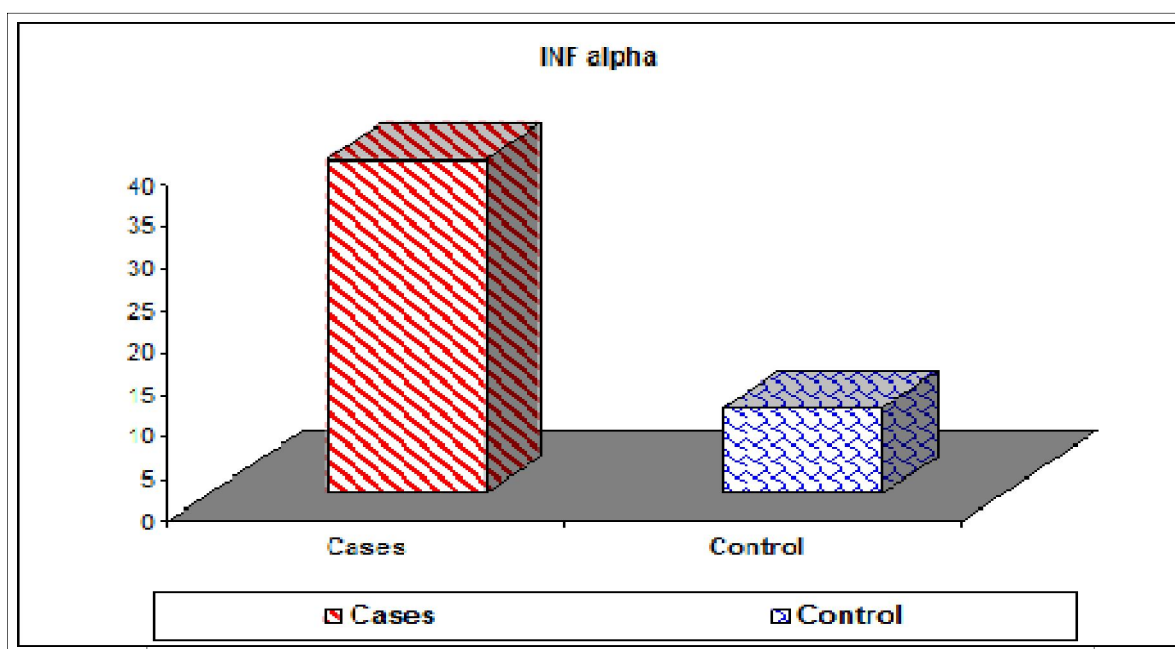
	Mean \pm SD		P-value
	Patients	Controls	
WBCs Thousands/cmm	3.0 \pm 0.87	7.65 \pm 1.81	0.001*
Hg gm/dl	9.00 \pm 1.02	13.21 \pm 0.65	0.001*
PLT Thousands/cmm	87.50 \pm 17.08	244.40 \pm 74.28	0.001*

Table (3): Comparison of biochemical characteristics among the studied groups

		Patients	Controls	P-value
ALT (U/L)	Mean \pm SD	51.65 \pm 2.68	28.335 \pm 2.11	0.001*
AST (U/L)	Mean \pm SD	58.22 \pm 1.99	30.2 \pm 3.75	0.001*
Albumin (g/dl)	Mean \pm SD	2.50 \pm 0.49	4.45 \pm 0.29	0.001*
T.B (mg/dl)	Mean \pm SD	1.12 \pm 0.19	0.77 \pm 0.16	0.001*

Table (4): Comparison of serum INF alpha between patients and controls

INF alpha	Cases	Control
Range	49.9 – 297.6	7.93 – 12
Mean \pm SD	106.90 \pm 48.51	10.20 \pm 1.20
T. test	238.288	
P. value	0.001*	

**Figure (1): Comparison of serum INF alpha between patients and controls.****Table (5): Comparison of TL 3 gene expression between patients and controls.**

TL 3 gene expression	Cases	Control
Range	0.005 – 1.585	1 – 1
Mean \pm SD	0.24 \pm 0.32	1.00 \pm 0.00
T. test	330.204	
P. value	0.001*	

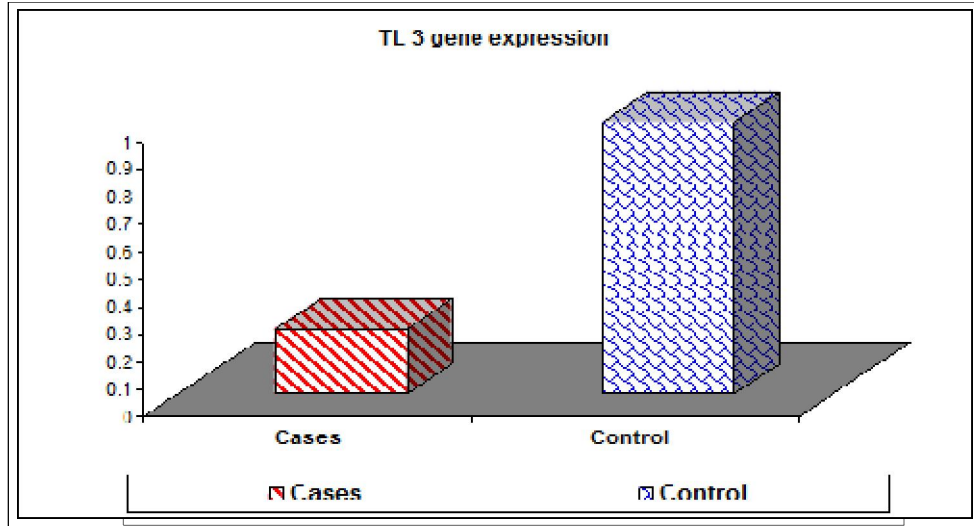


Figure (2): Comparison of TL 3 gene expression between patients and controls.

Table (6): Relation between TL 3 gene expression and (Age, and laboratory data in the studied groups)

With	TL 3 gene expression	
	r	P
Age	- 0.150	0.254
ALT	0.200	0.125
AST	- 0.032	0.807
ALB	0.167	0.108
Total Bilirubin	- 0.207	0.113
HB	0.137	0.297
PLT	0.018	0.893
TLC	0.090	0.493
INF alpha	0.902	0.001*

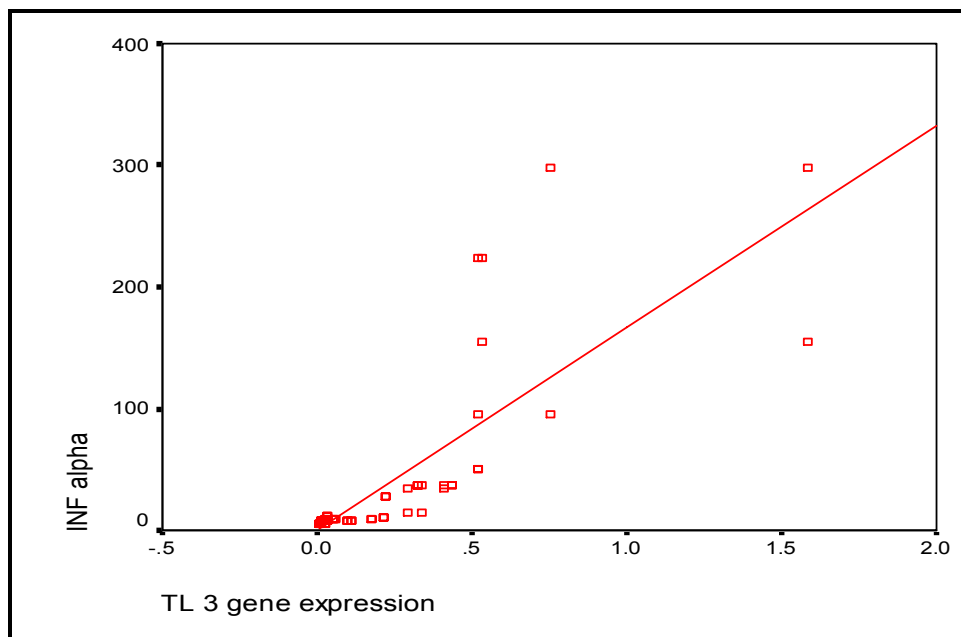


Figure (3). Correlation between TLR 3 gene expression and INF alpha level.

Discussion:

In Egypt hepatitis C is a significant public health problem where the prevalence is (14.7%) among clinical populations and groups at risk of exposure to infection the prevalence is even higher (*Mohamoud, et al., 2013*).

In the pathogenesis and progression of various inflammatory liver diseases as including autoimmune liver disease as fibrogenesis, and chronic HBV and HCV infection Toll-like receptors play an important (*Broering, et al., 2013*).

Viral double-stranded RNA (dsRNA) can be recognized by Toll-like receptor 3 (TLR3) which is a major intracellular receptor, therefore might influence the chronicity of such viruses, eventually leading to liver cirrhosis and hepatocellular carcinoma. Furthermore, polymorphisms in the TLR 3 gene have been associated with susceptibility to or the clinical progression of infection (*Wang et al., 2013*).

Our work revealed that there was down regulating in the expression level of TLR3 in patients as compared to normal healthy subjects. These results are in agreement with those of *Motavaf et al., (2014)*. *Firdaus et al., (2014)* indicate that in HCV patients there was more significant decrease in expression level of TLR 3 mRNA than individuals who had spontaneously cleared the viral infection.

On the other hand our result weren't in agreement with *Dolganic et al., (2006)* who stated that there was upregulation of almost all TLRs (including TLR3 and TLR7) in monocytes and lymphocytes of patients with chronic HCV infection.

This can be explained by a number of factors including difference in methodological approaches (cellular separation vs. total blood) and differences in clinical stage.

In our study the level of INF- α was significantly higher in patients as compared to control group. These results were coordinated with *Abdel-Raouf et al., (2014)* who cleared that the signaling pathway of TLR2, TLR4, TLR7 and TLR9 was inhibited by viral NS5A protein by binding to the adaptor protein MyD88 and inhibiting the recruitment of IRAK4 leading to a decrease in MyD88-dependent signals responsible for INF- α production.

The result of our study showed that there was significant correlation between TL 3 gene expression and INF alpha level in patients This goes with the result of *Mohammed et al., (2013)* who explained that there is strong correlation between expression level of INF alpha and TLR3 gene expression.

This is in agreement with *Atencia et al., (2007)* who documented that there is strong correlation in expression levels of TLR3 and TLR7 with the

expression level of INF- α .

Our study showed that there was no significant correlation between TLR 3 gene expression and (Age, ALT, AST, ALB, Total Bilirubin, HB, PLT and TLC) this is in agreement with *Mohammed et al., (2013)*.

Different IFN subtypes play an important role in hepatitis c therapy as they have different antiviral potencies against HCV, and some subtypes have synergistic activities in combination with others, TLR activation induces a range of different IFN subtypes. For example, the TLR7 agonist imiquimod has been shown to induce IFN- 1, - 2, - 5, - 6, and - 8 (*Puig et al., 2012*).

Authors:

Dalia El Sayed Sherief, Amaa. Emubark Hassan, Eman Mohamed Habeeb
Clinical pathology department, Faculty of Medicine, Kafr El-Sheikh University, Kafr El-Sheikh, Egypt,
Corresponding author: D.E. Sherief[†]; Elgeish St., Kafr El-Sheikh University hospitals, Kafr El-Sheikh, Egypt
Just2008@windowslive.com
Tel.: 20/010980330898

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