Association of Toll-like receptor (TLR) 3 & 9 genes' polymorphism with Hepatitis C virus-specific cell-mediated immunity outcome among Egyptian healthcare workers

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Abstract

Variations in immune responses could define successful resistance to Hepatitis C Virus (HCV) infection. Toll-like receptors (TLR)-3 are innate detectors of dsRNA viruses while TLR9 recognizes bacterial and viral unmethylated CpG motifs. We previously reported that TLR3.rs3775290 "CC" genotype was associated with HCV chronicity, while TLR9 gene played no major role in this infection. This study identified the role of TLR3.rs3775290 (c.1377C/T), TLR9.rs5743836 (-1237T-C) and TLR9.rs352140 (G2848A) gene polymorphisms in predicting the outcome of HCV-specific cell-mediated immunity (CMI) among Egyptian healthcare workers (HCWs) and patients. We enrolled 546 subjects (409 HCWs and 137 patients) divided into four groups. Group1: 265 seronegative, aviraemic subjects; group2: 25 seronegative, viraemic subjects; group3: 87 subjects with spontaneously resolved HCV infection; and group4: 169 chronic HCV HCWs and patients. All subjects were genotyped for TLR3.rs3775290, TLR9.rs5743836 and TLR9.rs352140 SNPs by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis. We, also, quantified HCV-specific CMI in 265 HCWs distributed among the four groups using an interferon gamma (IFN-γ) enzyme-linked immunospot (ELISpot) assay in response to nine HCV genotype 4a overlapping 15mer peptide pools covering the whole viral genome. No statistically significant difference was found between CMI responding subjects among Egyptian HCWs with different HCV states and TLR3.rs3775290 genotype or TLR9.rs352140. However, there was a significant relationship between the outcome of the HCV-specific CMI and the TLR9.rs5743836 genotype among the responding subjects (p=0.005) and the chronic HCV patients (p=0.044). In conclusion, TLR9.rs5743836 SNP; but not TLR3.rs3775290 or TLR9.rs352140 genotypes; could predict the outcome of HCV-specific CMI responses among genotype-4-infected Egyptians.

Association of Toll-like receptor (TLR) 3 & 9 genes' polymorphism with Hepatitis C virus-specific cell-mediated immunity outcome among Egyptian healthcare workers

Running title : TLR3&9 SNPs in HCV-infected Egyptians

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Abbreviations: CMI, Cell-mediated immunity; HCV, hepatitis C virus; HCWs, healthcare workers; TLR, toll-like receptor

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Summary:

Variations in immune responses could define successful resistance to Hepatitis C Virus (HCV) infection. Toll-like receptors (TLR)-3 are innate detectors of dsRNA viruses while TLR9 recognizes bacterial and viral unmethylated CpG motifs. We previously reported that TLR3.rs3775290 "CC" genotype was associated with HCV chronicity, while TLR9 gene played no major role in this infection. This study identified the role of TLR3.rs3775290 (c.1377C/T), TLR9.rs5743836 (-1237T-C) and TLR9.rs352140 (G2848A) gene polymorphisms in predicting the outcome of HCV-specific cell-mediated immunity (CMI) among Egyptian healthcare workers (HCWs) and patients. We enrolled 546 subjects (409 HCWs and 137 patients) divided into four groups. Group1: 265 seronegative, aviraemic subjects; group2: 25 seronegative, *viraemic* subjects; group3: 87 subjects with spontaneously resolved HCV infection; and group4: 169 chronic HCV HCWs and patients. All subjects were genotyped for TLR3.rs3775290, TLR9.rs5743836 and TLR9.rs352140 SNPs by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis. We, also, quantified HCV-specific CMI in 265 HCWs distributed among the four groups using an interferon gamma (IFN-γ) enzyme-linked immunospot (ELISpot) assay in response to nine HCV genotype 4a overlapping 15mer peptide pools covering the whole viral genome. No statistically significant difference was found between CMI responding subjects among Egyptian HCWs with different HCV states and TLR3.rs3775290 genotype or TLR9.rs352140. However, there was a significant relationship between the outcome of the HCV-specific CMI and the TLR9.rs5743836 genotype among the responding subjects (p =0.005) and the chronic HCV patients (p =0.044). In conclusion,

TLR9.rs5743836 SNP; but not TLR3.rs3775290 or TLR9.rs352140 genotypes; could predict the outcome of HCV-specific CMI responses among genotype-4-infected Egyptians.

Introduction

Variations in immune responses may help define successful resistance to Hepatitis C virus (HCV) infection particularly among seronegative healthcare workers, reviewed elsewhere [1]. Toll-like receptors (TLR)-3 are innate detectors of dsRNA of viruses while TLR9 recognizes bacterial and viral unmethylated CpG motifs. HCV virions bind to the cell surface and enter the cell via receptor-mediated endocytosis. The structure of HCV with different parts are recognized by different TLRs. The core and non-structural (NS) proteins are important sequences recognized by pattern recognition receptors (PRRs), including TLRs. They are, also, important inhibitors of TLR signalling [2, 3]. HCV core and NS proteins are important pathogen-associated molecular patterns (PAMPs) for TLR2, TLR3, TLR4, TLR7, 8, and 9. TLR3 is important for its antiviral immune effects, and TLR3 stimulated non-parenchymal liver cells are able to regulate HCV replication through production of interferon (IFN)-β [4, 5]. TLR3 mRNA is significantly increased in monocytes in chronic HCV infection [6]. An IFN-responsive element has been identified in the promotor region of the TLR3 gene, and it, therefore, seems likely that TLR3 expression is responsive to IFN treatment in HCV infection [7]. Myeloid dendritic cells (mDCs) have normal functioning TLR3 and can produce interleukin (IL)-12, IL-6, IL-10, IFN-γ, and tumour necrosis factor (TNF)-α with TLR3 stimulation despite HCV infection [8].

HCV viral proteins stimulate TLR signalling, which plays an important role in viral immune clearance. However, HCV can simultaneously evade immune clearance through specifically targeting and impairing TLR signalling through several mechanisms. First, HCV interferes with signalling via the TIR-domain-containing adapter-inducing IFNβ/TANK-binding kinase (TRIF)/ TBK)1- Interferon regulatory factor 3 (IRF3) pathway. The HCV NS3 protein induces degradation of TRIF, while the NS3/4A protein impedes IRF3 and NFκB activation by reducing the amount of TRIF in circulation and by generating cleavage products with dominant-negative activity [4, 9]. NS3/4A, also, interacts directly with TBK1 to reduce TBK1-IRF3 interaction and, therefore inhibit IRF3 activation [10]. HCV, also, interferes with the TLR-MyD88 (Myeloid differentiation primary response 88) pathway through NS5A interaction with MyD88 to prevent Interleukin-1 receptor associated kinase 1 (IRAK1) recruitment and cytokine production in response to ligands for TLR2, TLR4, TLR7, and TLR9 [11].

HCV has been shown to regulate TLR9 expression via transcription factor (Elk-1), which is an important signal integration point between T-Cell Receptor (TCR) and CD28 in T helper 1 (Th1) cell activation [12]. HCV also impairs TLR9-mediated IFN-α and IFN-β production and human leukocyte antigen DR (HLA-DR) expression by pDCs, associated with impaired activation of naïve T cells [13]. TLR9 signalling in mDCs is unaffected [8, 13]. It is, therefore, clear that compartmentalization of effects on TLR function is a key strategy by which HCV can evade immune clearance yet still lead to chronic inflammatory hepatic damage and liver fibrosis.

We previously showed that TLR3.rs3775290 "CC" genotype was associated with HCV chronicity, while TLR9 gene played no major role in HCV infection [14]. This study identified the role of TLR3.rs3775290 (c.1377C/T), TLR9.rs5743836 (-1237T-C) and TLR9.rs352140 (G2848A) single nucleotide polymorphisms (SNP) in predicting the outcome of HCV-specific cell-mediated immunity (CMI) among Egyptian health-care workers (HCWs) and patients. We show that TLR9.rs5743836 SNP; but not TLR3.rs3775290 or TLR9.rs352140 genotypes; could predict the outcome of HCV-specific CMI responses among genotype-4-infected Egyptians.

Subjects and Methods

Study subjects

Two hundred sixty-five HCWs were selected for the evaluation of IFN-γ producing T-cells by an Enzyme Linked Immuno-Spot (ELISpot) assay in response to nine HCV genotype 4a overlapping 15mer peptide pools

covering the whole viral genome. The study protocol was approved by the NLI-Institutional Review Board (IRB), and all subjects gave informed consent prior to enrolment. The demographic characteristics of the participants were previously reported [14].

Laboratory testing

HCV molecular diagnosis and quantification of HCV-RNA

HCV diagnosis was determined by using the Murex third generation anti-HCV ELISA assay (version 4.0 USA) that utilizes antigens from the core, NS3, NS4 and NS5 regions of the virus. All the study subjects were tested by quantitative real-time RT-PCR (qPCR) for detection of current HCV infection and quantification of the HCV viral load using QIAamp® Viral RNA purification kit (QIAGEN, USA). Amplification step was performed using AgPath-ID one-step assay according to the manufacturer's instructions (Applied Biosystems-Life Technologies Corporation, CA).

HCV-genotyping and genotyping for TLR3.rs3775290 (c.1377C/T) SNP

PCR-RFLP was used for HCV genotyping after reverse transcription as described [15].

PCR-RFLP was also performed to identify TLR3.rs3775290 (c.1377C/T) gene SNP. PCR was carried out in a total reaction volume of 25 µl with 20 pmol of each of the forward and reverse primers as described [16].

Genotyping for TLR9.rs5743836 (-1237T-C) and TLR9.rs352140 (G2848A) SNPs

PCR for TLR9.rs5743836 (-1237T-C) was carried out in a total reaction volume of 25 µl with 20 pmol of each of the forward and reverse primers as described [17]. On the other hand, PCR for TLR9.rs352140 (G2848A) was carried out in a total reaction volume of 25 µl with 20 pmol of each of the forward and reverse primers as described [16].

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An ELISPOT assay was used to quantify IFN-γ production by PBMC isolated from the study subjects in response to nine HCV genotype 4a isolate ED43 peptide antigen pools composed of 15 (15mer) and overlapping by 10 amino acids. These were 600 peptides combined in nine pools corresponding to all the HCV proteins. These synthetic peptides were custom synthesized by Mimotopes (Australia). Approximately 15ml of whole blood were collected into EDTA vacutainer tubes (Becton Dickinson Biosciences, NJ, USA). PBMC were isolated by Ficoll-Hypaque density gradient centrifugation and viability was determined by trypan blue exclusion method. Briefly, $2x10^5$ PBMC (200µl/well) were incubated in triplicate cultures in the ELISpot plates (Whatman Unifilter, USA) coated with anti-human IFNγ antibody and incubated for ˜16 hours with or without recombinant HCV antigens at 3µg/ml of each single peptide in complete RMPI-1640 medium. Negative and positive controls included medium containing DMSO alone and 0.1µg/ml of SEB or other polyclonal stimuli, respectively. At the end of the incubation period, the assay was developed until the appearance of spots, and then the wells were rinsed with tap water to stop the reaction. The number of spots per well was counted using an automated ELISpot reader (Cellular Technology Ltd., Cleveland, USA) as described [18]. The average number of spot forming cells (SFC) in control wells were subtracted from the average number of peptide-stimulated wells to correct for background cytokine production and are expressed as SFC/million PBMC. A positive HCV antigen-specific response was considered if the SFC in the presence of antigen were at least three-fold the number of SFC in the medium control and if there was >55 SFC/million PBMC, as we previously reported [19].

Statistical analysis

Allele and genotype frequencies of each SNP were determined by direct counting. Genotype and allele frequencies between groups were compared using the χ^2 test and Fisher's exact test when appropriate. For allelic associations, p values with Yates correction and odds ratio (OR) with 95% confidence intervals (CI) were calculated using Statcalc program (Epiinfo version 6.0.4, CDC, Atlanta, GA). Pair-wise LD (D', r^2) was estimated by use of Haploview software version 4.2. Chi-square tests were performed for categorical

data, while student's t -test (or Mann Whitney U test when appropriate) were performed for comparison of continuous data. Differences in means with a significance value ($p<0.05$) were considered significant. Standard errors (and/or deviations) of treatment means were also calculated.

Results

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All subjects enrolled into the study were tested for IFN-γ production by PBMCs using ELISpot assay to evaluate CMI response against nine HCV genotype 4a isolate ED43 peptide antigen pools composed of 15 (15mer) and overlapping by 10 amino acids. Only 140 (53%), 20 (80%), 35 (40%) and 70 (41.4%) samples had valid CMI data among the four study groups (seronegative, aviraemic subjects; seronegative, *viraemic*subjects; spontaneously resolved subjects and chronic HCV patients groups, respectively).

There was no relationship between the outcome of the HCV-specific immune response and the TLR3.rs3775290 genotype among the 263 HCWs with valid CMI responses and TLR3.rs3775290 genotyping tests (p= 0.222). Also, there was a significantly (p =0.004) higher proportion of the CC genotype (73.3%) in responding subjects than in non-responding seronegative, aviraemic subjects (65.1%). In addition, there was a differential distribution of the three TLR3.rs3775290 genotypes among the total subjects with and without HCV-specific CMI (Table 1).

The average totals (±SEM) of IFN-γ responses measured in SFC per million PBMCs in the *responding* total subjects were 455±90, 507±122, and 317±45 with the TLR3.rs3775290 genotype CC (n=37), CT (n=16) and TT (n=6). On the other hand, among the *non-responding* subjects, the average totals (±SEM) of IFN-γ SFC per million PBMCs in total subjects were 29±5, 23±5, and 49±19 with the TLR3.rs3775290 genotype CC (n=130), CT (n=65) and TT (n=9), respectively. There was no significant difference between the responding total subjects with different TLR3.rs3775290 genotypes (p =0.89; Figure 1A).

For the *seronegative, aviraemic* subjects, the average totals of HCV-specific IFN-γ SFC/10^6 PBMCs (±SEM) of the responding subjects were 404±97.7, 1058±807.5, and 249±21.9, among those with the TLR3.rs3775290 CC genotype (n=22), CT (n=4) and TT (n=4) genotypes, respectively. Differences were not statistically significant (p =0.88). On the other hand, the average totals of IFN-γ SFC/10^6 PBMCs (± SEM) of the non-responding subjects were 26.7±5.7, 16.7±4, and 13.3±13.3, among those with the TLR3.rs3775290 CC (n=71), CT (n=36) and TT (n=2) genotypes, respectively (Figure 1B).

Regarding *seronegative* , *viraemic* subjects, the average totals (±SEM) of HCV-specific IFN-γ SFC/10^6 PBMCs for*responding* subjects were 657±443, and 163, among those with the TLR3.rs3775290 genotype CC (n=3) and CT (n=1) genotypes, respectively. There were no responding subjects with the TT genotype. Differences were not statistically significant (p =0.18). On the other hand, the average totals of IFN-γ SFC/10^6 PBMCs (±SEM) of the non-responding subjects were 42±22, 30±11 and 8±8, among those with the TLR3.rs3775290 CC (n=9), CT (n=5) and TT (n=2) genotypes, respectively (Figure 1C).

For *spontaneously resolved subjects,* the average totals of HCV-specific IFN-γ SFC/10^6 PBMCs (±SEM) of the*responding* subjects were 793±605.8, and 325±121, among those with the TLR3.rs3775290 CC (n=2), CT (n=5) genotypes, respectively. No TT genotype was recorded in this category. Differences were not statistically significant (p =0.698). On the other hand, the average totals of IFN-γ SFC/10^6 PBMCs (±SEM) of the non-responding subjects were 51±21, 15±12 and 58, among those with the TLR3.rs3775290 CC (n=14), CT (n=12) and TT (n=1) genotypes, respectively (Figure 1D).

For *chronic HCV patients* , the average totals of HCV-specific IFN-γ SFC/10^6 PBMCs (±SEM) of the *responding*subjects were 439±214, 349 ±181, and 453±9, among those with the TLR3.rs3775290 CC (n=10), CT (n=6) and TT (n=2) genotypes, respectively. Differences were not statistically significant (p =0.228). On the other hand, the average totals of IFN-γ SFC/10^6 PBMCs (±SEM) of the non-responding chronic HCV patients were 26±8, 38±13, and 84±36, among those with the TLR3.rs3775290 CC (n=36), CT (n=13) and TT (n=4) genotypes, respectively (Figure 1E).

Relationship between the TLR9.rs5743836 (-1237T-C) genotype and the HCV-specific IFN ELISpot response

There was no relationship between the outcome of the HCV-specific CMI response and the TLR9.rs5743836 genotype among the 265 HCWs with valid CMI responses and TLR9.rs5743836 genotyping tests (Table 2,p= 0.12). However, there was a statistically significant (p =0.019) lower proportion of the TT genotype in responding spontaneously resolved subjects compared to non-responding spontaneously resolved subjects (20% Vs 80%, respectively; Table 2).

The average totals (±SEM) of IFN-γ responses measured in SFC per million PBMCs in the responding total subjects were 624±125, and 381±100 with the TLR9.rs5743836 CT (n=18) and TT (n=41) genotypes, respectively. There were no responding subjects with the CC genotype. On the other hand, among the non-responding subjects, the average totals (±SEM) of IFN-γ SFC per million PBMCs in total subjects were 40±23, 40±10, and 26±4, among those with the TLR9.rs5743836 CC (n=4), CT (n=40) and TT (n=162), respectively. There was a significant difference between the responding total subjects with different TLR9.rs5743836 genotypes (p =0.005; Figure 2A).

For the *seronegative, aviraemic* subjects, the average totals of HCV-specific IFN-γ SFC/10^6 PBMCs (±SEM) of the*responding* subjects were 617±192, and 398±164, among those with the TLR9.rs5743836 CT (n=10) and TT (n=20) genotypes, respectively. There were no responding subjects with the CC genotype. Differences were not statistically significant (p =0.159). On the other hand, the average totals of IFN-γ SFC/10^6 PBMCs (±SEM) of the non-responding seronegative, aviraemic subjects were 32±31, 44±14, and 18±3, among those with the TLR9.rs5743836 CC (n=3), CT (n=25) and TT (n=82) genotypes, respectively (Figure 2B).

For *seronegative* , *viraemic* subjects, the average totals of HCV-specific IFN-γ SFC/10^6 PBMCs (±SEM) of the*responding* subjects were 238, and 632±455, among those with the TLR9.rs5743836 CT (n=1) and TT (n=3) genotypes, respectively. There were no responding subjects with the CC genotype. Differences were not statistically significant (p =0.654). On the other hand, the average totals of IFN-γ SFC/10^6 PBMCs (±SEM) of the non-responding subjects were 65, 18±14, and 38±18, among those with the TLR9.rs5743836 CC (n=1), CT (n=4) and TT (n=11) genotypes, respectively (Figure 2C).

For *spontaneously resolved subjects* , the average total HCV-specific IFN-γ SFC/10^6 PBMCs (±) of the*responding* subjects was 795±345, and 206±49, among those with the TLR9.rs5743836 CT (n=3) and TT (n=4) genotypes, respectively. There were no responding subjects with the CC genotype. Differences were not statistically significant (p =0.077). On the other hand, the average totals of IFN-γ SFC/10^6 PBMCs (±) of*non-responding subjects* were 1, and 36±12, among those with the TLR9.rs5743836 CT (n=1) and TT (n=27) genotypes, respectively. Also, there were no subjects with the CC genotype among non-responding subjects (Figure 2D).

For *chronic HCV patients* , the average totals of HCV-specific IFN-γ SFC/10^6 PBMCs (± SEM) of the *responding*subjects were 610±212, and 354±156, among those with the TLR9.rs5743836, CT (n=4) and TT (n=14) genotypes, respectively. There were no responding subjects with the CC genotype. Differences were statistically significant (p =0.044). On the other hand, the average totals of IFN-γ SFC/10^6 PBMCs (±SEM) of the *non-responding chronic* HCV subjects were 43±19 and 32±8, among those with the TLR9.rs5743836 CT (n=10) and TT (n=42) genotypes, respectively. There were no subjects with the CC genotype among the non-responding chronic patients (Figure 2E).

Ρελατιονσηιπ βετωεεν τηε ΤΛΡ9.ρσ352140 (Γ2848Α) γενοτψπε ανδ τηε Η˝˝-σπεςιφις ΙΦΝ-γ ΕΛΙΣποτ ρεσπονσε

There was no relationship between the outcome of the HCV-specific CMI response and the TLR9.rs352140 (G2848A) genotype among the 258 HCWs subjects with valid CMI responses and TLR9.rs352140 genotyping tests (p= 0.356). Also, there was no significant difference (p= 0.299) in the proportion of the GG genotype in responding (46.4 %) and in the non-responding seronegative aviraemic subjects (31.4 %; Table 3).

The average totals (±SEM) of IFN-γ responses measured in SFC per million PBMCs in the responding total

subjects were 587±176, 390±85, and 327±45, among those with the TLR9.rs352140 GG (n=24), GA (n=23) and AA (n=10) genotypes, respectively. On the other hand, among the non-responding subjects, the average totals (±SEM) of IFN-γ SFC per million PBMCs in the total subjects were 30±6, 31±6, and 26±7, among those with the TLR9.rs352140 (G2848A) GG (n=66), GA (n=86) and AA (n=49) genotypes, respectively. There was no significant difference between the responders of the total subjects with the TLR9.rs352140 genotypes (p =0.652; Figure 3A).

For the *seronegative, aviraemic* subjects, the average totals of HCV-specific IFN-γ SFC/10^6 PBMCs (±SEM) of the*responding* subjects was 672±278.8, 307±86, and 357±68, among those with the TLR9.rs352140 GG (n=13), GA (n=10) and AA (n=5) genotypes, respectively. Differences were not statistically significant (p =0.594). On the other hand, the average totals of IFN-γ SFC/10^6 PBMCs (±SEM) among the *non-responding seronegative, aviraemic* subjects were 23±7, 31±8, and 17±5, among those with the TLR9.rs352140 GG (n=33), GA (n=43) and AA (n=29) genotypes, respectively (Figure 3B).

For *seronegative, viraemic* subjects, the average totals of HCV-specific IFN-γ SFC/10^6 PBMCs (±SEM) of the responding subjects were 214±24.2, and 853±689, among those with the TLR9.rs352140 GG (n=2) and GA (n=2) genotypes, respectively. There were no responding subjects with the AA genotype. Differences were not statistically significant (p =1.000). On the other hand, the average totals of IFN-γ SFC/10^6 PBMCs (±SEM) were 12±2.22, 30±10.4, and 60±47.6, among the *non-responding seronegative* , *viraemic* subjects with the TLR9.rs352140 (G2848A) GG (n=3), GA (n=9) and AA (n=4) genotypes, respectively (Figure 3C).

For *spontaneously resolved subjects* , the average totals of HCV-specific IFN-γ SFC/10^6 PBMCs (±SEM) of the*responding* subjects were 244±48.2, and 619±301, among those with the TLR9.rs352140 GG (n=3) and GA (n=4) genotypes, respectively. There were no responding subjects with the AA genotype. Differences were not statistically significant (p =1.000). On the other hand, the average totals of IFN-γ SFC/10^6 PBMCs (±SEM) were 26±14.1, 60±26.9, and 10±10, among the *non-responding spontaneously resolved subjects* with the TLR9.rs352140 GG (n=11), GA (n=10) and AA (n=7) genotypes, respectively (Figure 3D).

For *chronic HCV patients* , the average totals of HCV-specific IFN-γ SFC/10^6 PBMCs (±SEM) of the responding subjects were 698±357.2, 245±51.5, and 296±57.8, among those with the TLR9.rs352140 GG (n=6), GA (n=7) and AA (n=5) genotypes, respectively. Differences were not statistically significant (p =0.775). On the other hand, the average totals of IFN-γ SFC/10^6 PBMCs (±SEM) of *non-responding chronic HCV subjects* were 46±13.8, 18±7.9, and 50.9±23.1, among those with the TLR9.rs352140 GG (n=19), GA (n=24) and AA (n=9) genotypes, respectively (Figure 3E).

Discussion

In this study, there was no statistically significant difference between CMI responding subjects in Egyptian HCWs with different HCV states with different TLR3.rs3775290 (c.1377C/T) genotypes. Also, there was no relationship between the outcome of the HCV-specific CMI response and the TLR3.rs3775290 (c.1377C/T) genotype among the 263 subjects tested. This agrees with another report [20]. The role of TLR3 was investigated in four different infectious viral models including lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), Murine cytomegalovirus (MCMV), and reovirus in TLR3$^{-/-}$ mice. The investigators found that TLR3 is not always required for the generation of effective antiviral responses, as the absence of TLR3 did not alter either viral pathogenesis or host's generation of adaptive antiviral responses to those viruses. Interestingly, intracellular transduction of poly I:C initiates activation of an IFN response in a TLR3-independent manner, thus limiting the role of TLR3 in the IFN pathway [21]. Other reports [22, 23] indicate that HCV may use TLR3 pathway to evade immune surveillance via HCV NS3/4A protease-mediated cleavage of the TLR3 adaptor protein TRIF. Also, HCV NS3/4A interferes with RIG-I, a key factor in TRIF-independent signalling [21]. In addition, it was suggested that several polymorphisms that alter TLR3 amino acids initiate resultant changes in the protein and that they might downregulate the gene expression and lower the activities of TLR3 required for proper signalling [24]. Reduced activity of TLR3 results in failure to recognize invading microorganisms and insufficient immune responses, thus increasing

the likelihood of infections and infectious diseases [25].

Our data provide strong indirect evidence that TLR9 might play a greater role in HCV infection than previously expected. We identified an association of the polymorphism TLR9.rs5743836 within the TLR9 gene with the natural course of HCV infection. In this study, there was a strong relationship between the outcome of the HCV-specific CMI response and the TLR9.rs5743836 (-1237T-C) genotype among the 265 subjects with valid CMI responses in responders of total subjects of HCWs (p =0.005). Also, there was a statistically significant difference between responding subjects in chronic HCV patients with different TLR9.rs5743836 ($p = 0.044$).

We show that the TLR9-1237T allele was significantly associated with HCV-specific CMI response. In this regard, the TT genotype is transcribed more effectively than the CC genotype as reported by others [26] who showed that the wild-type construct elicits higher transcriptional activity compared to the variant CC allele. Data showed that subjects with the "favourable" TT allele would have HCV-specific CMI response when compared to those with the "unfavourable" CC allele. Also, several reports found that the mutant allele "T" imparts the immunity against the various pathogens. However, statistically inconsiderable, the mutant allele was declared to be linked with the depressed microbial load in Africans [27-29]. In addition, the mutant allele T manipulate immunity against establishing the infections, which is convincing that the TLR9 gene had bear the influence of genetic assortment to cope with the infections [30]. In contrast, functional analyses of the impact of the TLR9 polymorphisms on basal promoter activity revealed that the rs5743836 SNP provoke higher gene expression compared with the wild type promoter. Increased promoter activity of TLR9.rs573836 might be explained by the findings of others [31], who showed that the variant C allele at rs5743836 creates a potential NF-ϰB-binding site that increased the transcriptional activity of the gene. The presence of this extra putative NF-ϰB-binding site promotes TLR9 transcription in response to various stimuli more effectively than the wild type TLR9.rs5743836T sequence. This finding agrees with another report [32], TLR9 promoter SNPs are associated with the natural course of HCV infection and show higher transcriptional activities and imply the DNA sensor TLR9 in natural immunity against the RNA virus, HCV. Our data are analogous to another study [1, 33], where the rs5743836 TT variant has been shown to have a higher promoter activity than the CC genotype. That study suggested that this activity could result in an increased pro-inflammatory cytokine production during malaria infection leading to successful control and elimination of malaria parasites [33].

There was no relationship between the outcome of the HCV-specific immune response and the TLR9.rs352140 (G2848A) genotype among the 258 Egyptian HCWs subjects with valid CMI responses and TLR9.rs352140 genotyping. To date, there have been no reports showing an association between TLR9.rs352140 (G2848A) and HCV infection. The region around 2848 is the major coding region of the TLR9 protein [34, 35] and a polymorphism of the TLR9 2848 GA genotype has been reported to reduce TLR9 expression at the transcriptional level [35, 36]. Down regulated TLR9 could reduce the functions of the innate immune reactivity against HCV infection. Our data are analogous to another study which suggest that the TLR9 gene may play a role in cervical carcinogenesis but have a lesser (or no) role in tumour progression where subjects with the favourable" GG allele were reported to be more frequent than the "unfavourable" AA allele [37]. In addition, a recent meta-analysis demonstrated that there is no association between TLR9.rs352140 (G2848A) and cervical cancer susceptibility [38]. Our findings suggest that the TLR9.rs352140 (G2848A) genotype does not affect the natural course of HCV infection.

In conclusion, this study shows that TLR9.rs5743836 SNP; but not TLR3.rs3775290 or TLR9.rs352140 genotypes; could predict the outcome of HCV-specific CMI responses among genotype-4-infected Egyptians.

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Author contributions :

Conceptualization and design of experiments: SFA, SH, AO, ZZ, ER, and IW; Data collection, experimentation, analysis, and investigation: SH, SFA, MA, ZZ, IG, MS, WA; Funding acquisition and resources: SFA,

ER, and IW; Writing-original draft (SH and SFA); Writing-review and editing (SFA, AO, MS, MH, WA, MA, ER, and IW). All authors approved the final version of the manuscript.

Compliance with Ethical Standards :

Conflict of Interest: None declared.

Data Sharing and Data Accessibility: All data pertinent to this article are included herein.

Research involving human participants and/or animals :

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments.

Informed Consent : Informed consent was obtained from all enrolled study participants.

Table (1): Relationship between TLR3.rs3775290 (c.1377C/T) and HCV-specific cell mediated immune (CMI) response among NLI HCWs in terms of CMI responders frequencies.

p-value	Total	**TLR3.rs3775290 genotype**	**TLR3.rs3775290 genotype**	**TLR3.rs3775290 genotype**	**C**
		TT	**CT**	**CC**	
0.004	30 (21.6)	4 (13.3)	4 (13.3)	22 (73.3)	I
	109 (78.4)	2 (1.8)	36 (33)	71 (65.1)	N
0.694	4 (20)	0(0)	1 (25)	3 (75)	I
	16 (80)	2 (12.5)	5 (31.3)	9 (56.3)	N
0.425	7 (20.6)	0(0)	5 (71.4)	2 (28.6)	I
	27 (79.4)	1 (3.7)	12 (44.4)	14 (51.9)	N
0.57	18 (25.7)	2 (11.1)	6 (33.3)	10 (55.5)	I
	52 (74.3)	4 (7.7)	12 (23.1)	36 (69.2)	N
0.222	**59 (22.3)**	**6 (10.2)**	**16 (27.1)**	**37 (62.7)**	**I**
	204 (77.7)	**9 (4.4)**	**65 (31.9)**	**130 (63.7)**	N

Table (2) : Relationship between TLR9.rs5743836 (-1237T-C) and HCV-specific cell mediated immune (CMI) response among NLI HCWs in terms of CMI responders frequencies.

p-value	Total	**TLR9.rs5743836 genotype**	**TLR9.rs5743836 genotype**	**TLR9.rs5743836 genotype**	**C**
		TT	**CT**	**CC**	
0.353	30 (21.4)	20 (66.7)	10 (33.3)	0 (0)	I
	110 (78.5)	82 (74.5)	25 (22.7)	3 (2.7)	N
0.87	4 (20)	3 (75)	1 (25)	0 (0)	I
	16 (80)	11 (68.8)	4 (25)	1 (6.3)	N
0.019	7 (20)	4 (57.1)	3 (42.9)	0 (0)	I
	28 (80)	27 (96.4)	1 (3.6)	0 (0)	N
0.74	18 (25.7)	14 (77.8)	4 (22.2)	0 (0)	I
	52 (74.3)	42 (80.8)	10 (19.2)	0 (0)	N
0.12	**59 (22.3)**	**41 (69.5)**	**18 (30.5)**	**0 (0)**	I
	206 (77.7)	**162 (78.6)**	**40 (19.4)**	**4 (1.9)**	N

Table (3) : Relationship between TLR9.rs352140 (G2848A) and HCV-specific cell mediated immune (CMI) response among NLI HCWs in terms of CMI responders frequencies.

p-value	Total	TLR9.rs352140 genotype	TLR9.rs352140 genotype	TLR9.rs352140 genotype	GRO
		AA	**GA**	**GG**	
0.299	28 (21.1)	5 (17.9)	10 (35.7)	13 (46.4)	Respo
	105 (78.9)	29 (27.6)	43 (41)	33 (31.4)	Non-r
0.32	4 (20)	0 (0)	2 (50)	2 (50)	Respo
	16 (80)	4 (25)	9 (56.3)	3 (18.8)	Non-r
0.299	7 (20)	0 (0)	4 (57.1)	3 (42.9)	Respo
	28 (80)	7 (25)	10 (35.7)	11 (39.3)	Non-r
0.627	18 (25.7)	5 (27.8)	7 (38.9)	6 (33.3)	Respo
	52 (74.3)	9 (17.3)	24 (46.2)	19 (36.5)	Non-r
0.35	**57 (22.1)**	**10 (17.5)**	**23 (40.4)**	**24 (42.1)**	**Resp**
	201(77.9)	**49 (24.4)**	**86 (42.8)**	**66 (32.8)**	**Non-**

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Figure legends :

Figure (1) : **Relation between TLR3.rs3775290 (c.1377C/T) genotype and total HCV-specific CMI response among total subjects (A), seronegative, *aviraemic* subjects (B), seronegative*viraemic* subjects (C), spontaneously resolved subjects (D),and chronic HCV patients (E).** Data are shown as scatter plots. For each subject, the total cumulative number of HCV-specific IFN-γ spot-forming cells (SFC) per 10^6PBMCs is shown for responding and non-responding healthcare workers (HCWs) and patients. Error bars represent the SEM.

Figure (2) : **Relationship betweenTLR9.rs5743836 (-1237T-C) genotype and total HCV-specific CMI response among the total subjects (A), seronegative *aviraemic* subjects (B), seronega-**

tive*viraemic* subjects (C), spontaneously resolved subjects (D),and chronic HCV patients (E). Data are shown as scatter plots. For each subject, the total cumulative number of HCV-specific IFN-γ spot-forming cells (SFC) per 10^6PBMCs is shown for responding and non-responding healthcare workers (HCWs) and patients. Error bars represent the SEM.

Figure (3): Relationship between TLR9.rs352140 (G2848A) genotype and total HCV-specific CMI response among the total subjects (A) seronegative, *aviraemic* subjects (B) seronegative,*viraemic* subjects (C) spontaneously resolved subjects (D) and**chronic HCV patients (E).** Data are shown as scatter plots. For each subject, the total cumulative number of HCV-specific IFN-γ spot-forming cells (SFC) per 10^6 PBMCs is shown for responding and non-responding HCWs and patients. Error bars represent the SEM.

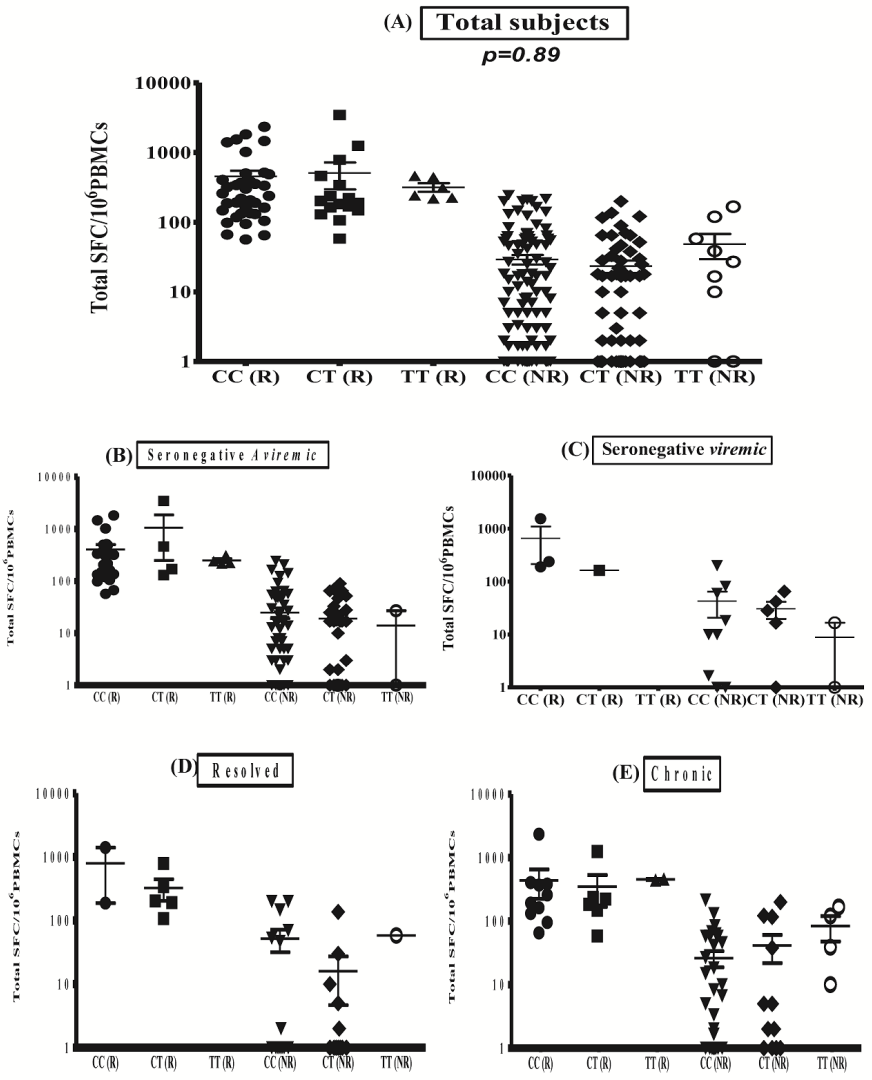

Figure 1:

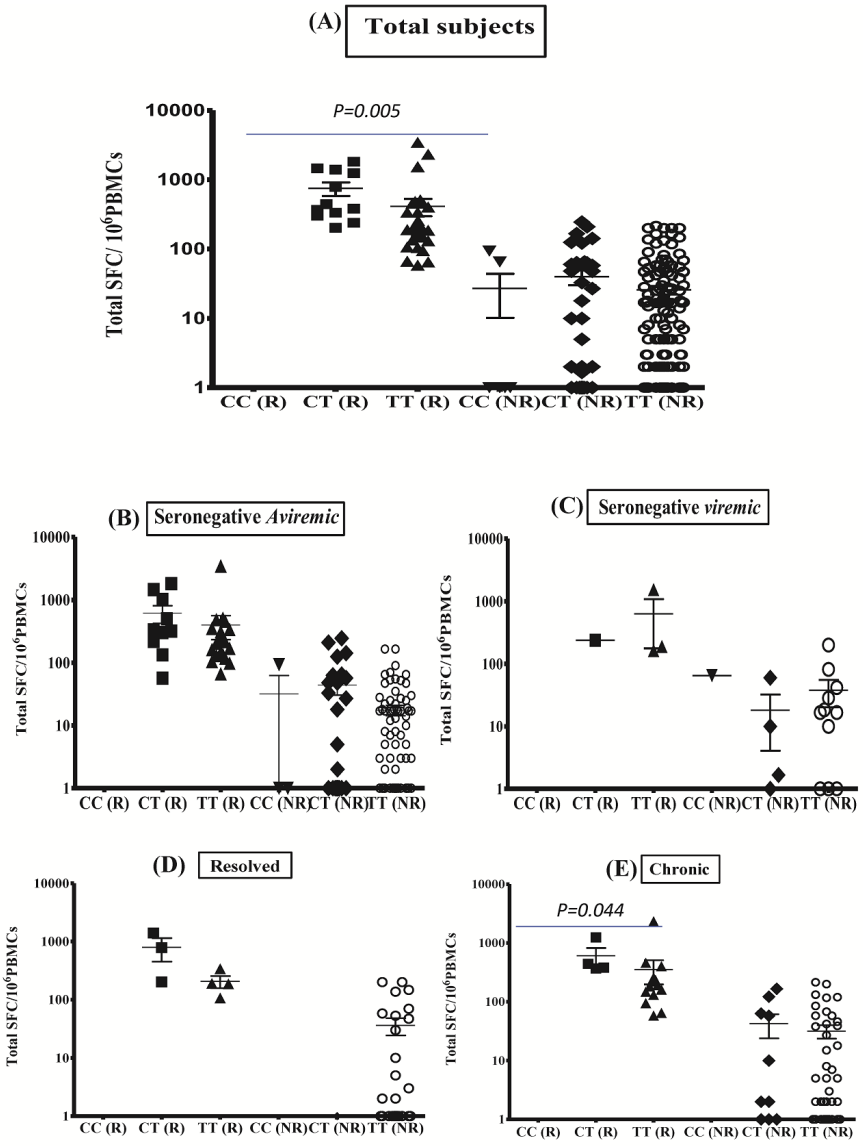

Figure 2:

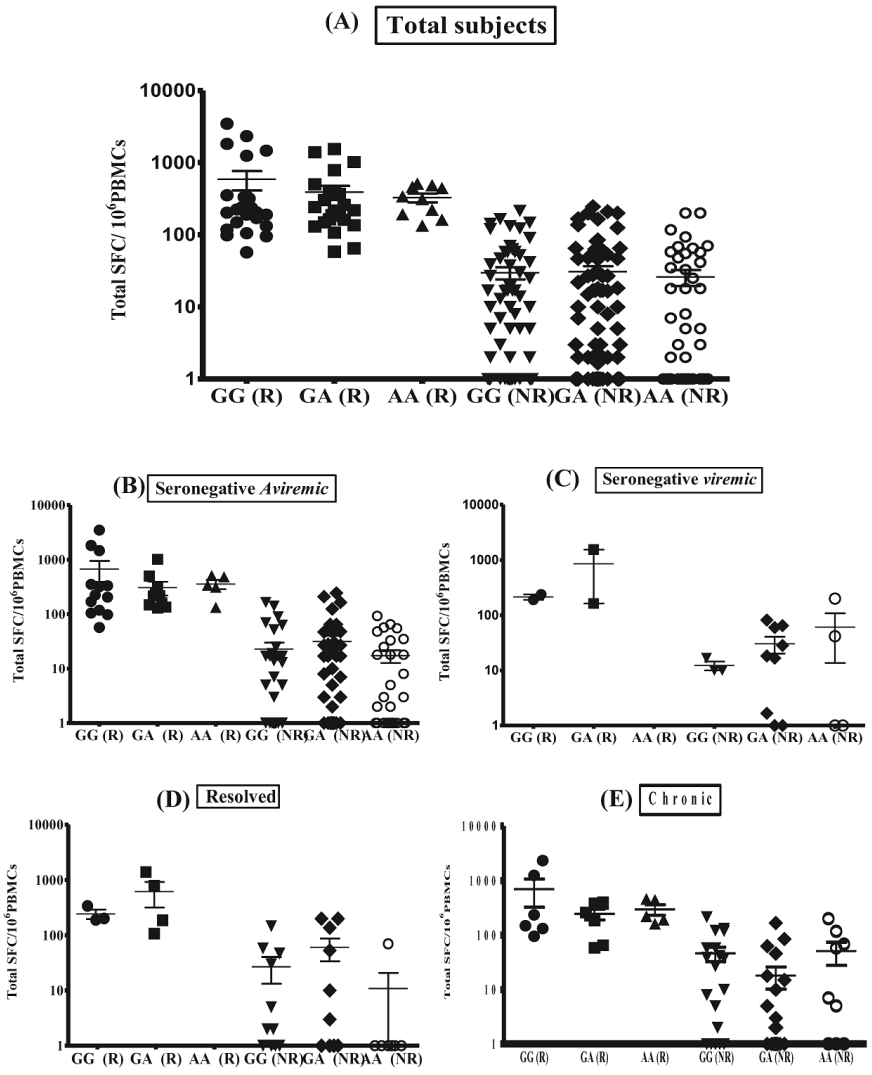

Figure 3: