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Original Article

Single nucleotide polymorphisms of interleukins associated with hepatitis C virus infection in Egypt

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Abstract

Introduction: Hepatitis C is a liver disease caused by the hepatitis C virus (HCV). It can cause both acute and chronic hepatitis infection. Based on secretion of required cytokines upon infection, HCV can improve its own RNA and successfully complete the replication cycle. Importantly, single nucleotide polymorphisms (SNPs) are the most common type of genetic variation and have been found to play a critical role in modulation of cellular cytokine production and interaction.

Methodology: A total of 100 blood samples were obtained from HCV patients, and 120 samples were obtained from healthy individuals who served as controls. SNPs of interleukin-10/592 (IL-10/592) and IL-4/589 were investigated for possible connection with HCV infection. Relative expression of IL-4, IL-6, and IL-10 were detected using real-time polymerase chain reaction and enzyme-linked immunosorbent assay

Results: The polymorphisms of IL-10 revealed a high rate of mutant genotype CC within the location IL-10/592 in HCV patients and controls, which resulted in low secretion of IL-10. Interestingly, the findings here demonstrate a positive association between HCV load of viremia and the mutant genotype IL-4-589/TT accompanied with low expression IL-4 in comparison with IL-6 expression.

Conclusions: These data suggest that the expression of IL-4 is inversely proportional to HCV load of viremia, and this connection is due to the high level of mutant genotype IL-4-589/TT in infected patients located in gene promoter and inhibits gene expression.

Key words: single nucleotide polymorphism; SNP; interleukins; hepatitis C virus.

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Introduction

More than 150 million people worldwide have chronic hepatitis C infection, and a significant number of patients suffer from liver cirrhosis or liver cancer. In Egypt, more than 11 million persons have been infected with this virus, which is accompanied by a high chance of liver cancer [1]. Typically, cellular immune response, which includes the production of endogenous interferons (IFNs) and cytokines connected with specific cellular pathways, is very important in recovery from acute hepatitis infection. Hepatitis C virus (HCV) infection induces a variety of intracellular signaling pathways that are either antiviral or are required to ensure efficient viral replication. A major cellular antiviral mechanism is the activation of the cascade that produces the type I IFNs such as IFN-α and IFN-β [2]. Host cell signaling pathways that support viral replication, such as Raf/MEK/ERK and mTor signaling cascade, are also activated during infection, followed by initiation of autophagic machinery in infected cells [3-5]. On the other hand, interleukins are large group of cellular cytokines that can modulate cellular immune response. Like other cytokines, interleukins are rapidly secreted from host cells in response to viral infection and environmental stress in order to trigger cascade signaling of neighbor cells. Some interleukins as well as IFNs induce tyrosine phosphorylation of STAT1 and other similar transcriptional factors [6]. For instance, IL-6 activates interferon regulatory factor 1 (IRF-1), which are growth-stimulating factors in B-hybridoma cells and growth-inhibiting factors in cancer cells [7,8]. Additionally, IL-10 has the ability to regulate the differentiation and function of T cells, moncytes, and macrophages, and subsequently controls the synthesis of cytokines including IL-2, IL-3, IL-12, tumor necrosis factor (TNF), and interferon gamma [9,10]. IL-10 plays a key role with anti-inflammatory effects during viral replication by limiting the immune response and preventing host damage [11,12]. Recently, evidence has indicated that the expression of IL-10 in different immune cells is regulated by various molecular events, including epigentic modifications, transcription factor interaction, gene activation, and signal transduction [13]. IL-4 is another cellular cytokine that endorses the

conversion of T helper cells (Th) to T helper type 2 cells (Th2), which produce more IL-4 as a feedback response [14]. The biological function of IL-4 includes regulation of humoral immunity and adaptive immunity via stimulation of T-cell proliferation and activation of B lymphocytes. IL-4 also has the ability to decease the production of Th1 cells, macrophages, IFN gamma, and cellular IL-12 [15]. HCV infection stimulates the activation of RNA helicase retinoic acid-inducible gene I (RIG-I), the cellular sensor for viral RNA, and IRFs, AP-1, and NF-κB transcription factors. These transcription factors are involved in signal transduction of toll-like receptors (TLRs) and transcription of anti-inflammatory cytokines and IFNs, including IFN-α and IL-1β [16]. On the other hand, single nucleotide polymorphisms (SNPs) play a critical role in the alternation of biological function of different IFNs and cytokines [17]. SNPs are the variations in genomic DNA sequences that reveal the possible differences between single nucleotides within a biological population [18]. Several studies indicate the association between HCV infection and cytokine gene polymorphisms. Similarly, the clinical outcome of HCV infection was associated with genotype polymorphisms of IL-2 (330/TT) and IL-10 (1082/AA). Abnormal serum alanine aminotransferase (ALT) level was associated with genotype polymorphisms of IL-10 (592/AC) and IL-4 (589/CC) [18]. Two genotype polymorphisms of IL-18 (137/GC and 607/CA) were associated with the outcome of HCV infection in Tunisian patients [20]. The analysis of IL-10 gene polymorphisms indicated a variation between the pro-inflammatory and anti-inflammatory cytokine responses, which may have resulted in immunodeficiency to HCV infection in Pakistan [21]. Meanwhile, no significant correlation has been found between SNPs of IL-10 and histological activity index in HCV patients [22]. Accordingly, in the current study, we further investigated the possible SNPs in IL-10 and IL-4 that may occur in samples collected from Egyptian patients.

Methodology

Samples condition

A total of 100 blood samples were collected from HCV-infected individuals (42 male and 58 female), in addition to 120 samples obtained from healthy individuals who served as controls (54 male and 66 female). HCV-infected patients at hepatic centers (private and public) in Egypt with at least three years of infection during 2013 were divided into the indicated two groups (male and female). All HCV-infected patients with positive evidence of infection using real-time polymerase chain reaction (PCR) had not received any treatment for HCV before participating in this study. Meanwhile, the healthy control groups were randomly and geographically matched, and tested negative for HCV antibodies.

Clinical diagnosis of HCV viremia

Whole-blood samples were investigated for HCV infection and then divided into two major groups: HCV-infected patients and healthy controls. Total RNA was isolated from each sample, and levels of HCV RNA were detected using quantitative real-time PCR using an HCV RG RT-PCR Kit (Artus, Qiagen, Valencia, USA) per the manufacturer's instructions.

Gene amplification and resection enzyme digestion

Blood samples were collected in sodium citrate sterile tubes. Genomic DNA was isolated using a Blood Genomic Prep Mini Spin Kit (GE Healthcare Life Science, Cairo, Egypt). Then the purified genomic DNA was used to amplify IL-10-592 and IL-4/589 genes using the specific primer sequences (F: 5'-CCTAGGTCACAGTGACGTGG-3', R: 5'-GGTGAGCACTACCTGACTAGC-3' and F: 5'-ACTAGGCCTCACCTGATACG-3', R: 5'-GTTGTAATGCAGTCCTCCTG-3', respectively) [19]. PCR was carried out in an AmpGene DNA thermal cycler and the following PCR parameters were used: initial denaturation at 95°C for 2 minutes, then 35 cycles at 94°C for 40 seconds, 60°C for 45 seconds, and 72°C for 60 seconds to amplify IL-10/592 and 94°C, 57°C, and 72°C for 30 seconds to amplify IL-4/589. The RsaI (Bio Basic, New York, USA) was used to digest IL-10-A592, while BsmFI (New England Bio Labs, Ipswich, UK) was used to digest IL-4-C589 [19].

ELISA

For quantitative measurement of individual interleukin, sandwich enzyme-linked immunosorbent assay (ELISA) was used to determine serum concentrations of IL-4, IL-6, and IL-10 using human ELISA kits (Abcam, 100570, Cambridge, USA; (Abcam, 46042, Cambridge, USA; and Abcam 46034, Cambridge, USA, respectively). Standards and samples were loaded into the 96-well plates and present interleukin was bound to the wells by the immobilized antibody. After washing, biotinylated interleukin antibody was added followed by horseradish peroxidase-conjugated streptavidin antibody. Finally, a TMB substrate solution was added to the wells and color developed based on the amount of interleukin

bound. The intensity of the color was measured at 450 nm.

RNA isolation and qRT-PCR

Total RNA from blood samples were isolated using TriZol (Invitrogen, Valencia, USA), and cDNA was generated from total RNA using a QuantiTech Reverse Transcriptase Kit (Qiagen, Valencia, USA) based on the manufacturer's protocol. The relative expression of IL-4, IL-6, and IL-10 were detected using a QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, USA) and oligonucleotides specific for each individual gene (Table 1). Levels of GAPDH were amplified using specific oligonucleotides and used for normalization.

Statistical analysis

Microsoft Excel was used for statistical calculation, graphs, and histograms. The frequencies of different alleles and genotypes were compared using a Chi-squared test. SDS 2.2.2 software was used to analyze the qRT-PCT data to drive the ΔΔCt values using the following equations; (ΔCt) = Ct-sample – Ct-control. (ΔΔCt) = ΔCt – ΔCt- normalized control. Lastly, the relative gene expression is equal $2^{-\Delta\Delta ct}$ of the final values [23,24].

Results

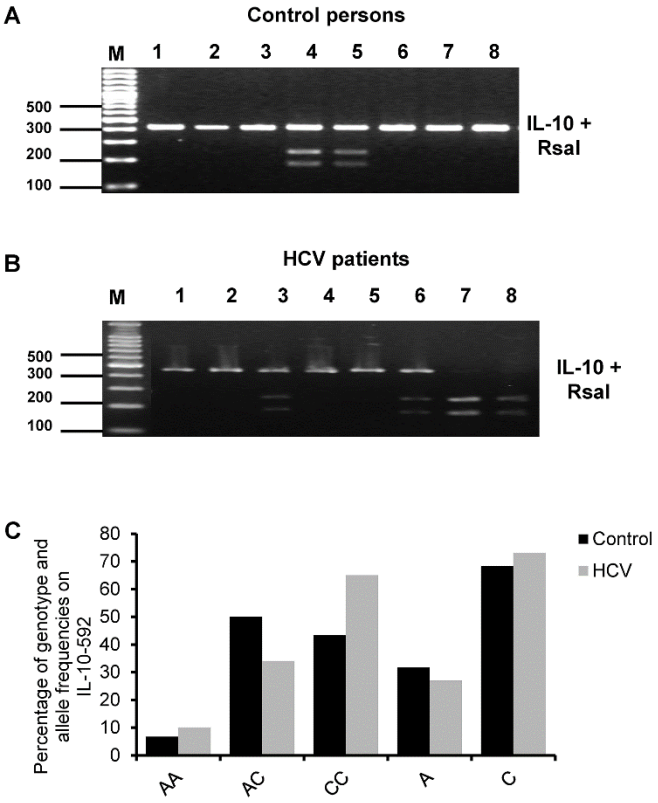
IL-10-592/CC genotype is particularly increased in HCV infected patients

Three different genotypes within the location 592 of IL-10 were identified: the wild genotype AA, and the mutant genotypes AC and CC. Based on the frequency of A and C alleles in this location, the IL-10 gene was amplified using specific primers and genomic DNA isolated and purified from HCV-infected patients and healthy controls. Subsequently, isolated IL-10 fragments were individually digested with Rsa1 enzyme and electrophoresed in agarose gel to monitor the location of different segments. Control and patient samples revealed the different genotypes of IL-10 with various frequencies (Figures 1A and 1B). Interestingly,

Table 1. Oligonucleotide sequences used for detection of indicated genes at RNA level.

Description	Primer sequences 5'-3'
IL4-For	AGAAGACTCTGTGCACCGAGTTGA
IL4-Rev	CTCTCATGATCGTCTTTAGCCTTT
IL6-For	CGAAAGTCAACTCCATCTGCC
IL6-Rev	GGCAACTGGCTGGAAGTCTCT
IL10-For	CCCTGGGTGAGAAGCTGAAG
IL10-Rev	CACTGCCTTGCTCTTATTTTCACA
GAPDH-For	TGG CAT TGT GGA AGG GCT CA
GAPDH-Rev	TGG ATG CAG GGA TGA TGT TCT

Figure 1. IL-10-592 A/C SNP in HCV patients and controls. **(A)** Control samples reveal the different genotype polymorphisms indicated by wild genotype AA, lanes 1, 2, 3, 6, 7, and 8 in addition to the mutant genotype AC, lanes 4 and 5. **(B)** Patient samples indicate the wild genotype AA in lanes 1, 2, 4, and 5 in addition to the mutant genotypes AC in lanes 3 and 6 and CC in lanes 7 and 8. **(C)** The percentage of genotypes and alleles frequencies in both control and patient samples.



the frequency of different genotypes showed marginally significant values of the CC mutant genotype in patient samples compared with control samples (p = 0.55) (Table 2) (Figure 1C). Generally, the control samples comprised almost 6.7% wild genotype AA, 50% mutant genotype AC, and 43.3% mutant genotype CC (Table 1), whereas patient samples comprised approximately 10% wild genotype AA, 34% mutant genotype AC, and 56% mutant genotype CC (Table 2). Furthermore, the comparison between male and female in both control and patient samples showed negligible frequency differentiation of indicated genotypes (Table 3). These results indicate that the mutant genotype CC within the location 592 of IL-10 existed in HCV patients, but cannot be used as an indicator for HCV infection.

The IL-4-589/TT mutant genotype is significantly increased in HCV patients

Three different genotypes within the location 589 of IL-4 were identified: the wild genotype CC, and the

Table 2. Polymorphism frequencies of IL-10-592 in control and patient populations.

IL-10-592	HCV patients (n = 100)		Control persons (n = 120)		P
	No	%	No	%	
AA	10	10.0	8	6.7	**0.369**
AC	34	34.0	60	50.0	
CC	56	56.0	52	43.3	0.055
A	54	27.0	76	31.7	
C	146	73.0	164	68.3	0.285
Wild phenotype AA	10	10.0	8	6.7	0.369
Mutant phenotype (AC and CC)	90	90.0	112	93.3	0.463

mutant genotypes CT and TT. Based on the frequency of C and T alleles in this location, the IL-4 gene was amplified using specific primers and the products were digested with BsmF1 endonuclease. The final products were loaded in agarose gel to detect the location of different fragments that reveal the genotype polymorphisms of IL-4 at the position 589. The control samples revealed the different genotypes polymorphisms of IL-4 with various frequencies (Figure 2A). Interestingly, frequency of the mutant genotype TT was strongly reduced in control samples (Figure 2C). The patient samples revealed only the mutant genotypes CT and TT (Figure 2B). Furthermore, the wild genotype polymorphism CC completely disappeared in patient samples. Importantly, the mutant genotype TT was dramatically increased in patient samples compared with control samples (Figure 2C). Moreover, the control samples comprised almost 26.7% wild genotype CC, 70% mutant genotype CT, and 3.3% mutant genotype TT (Table 4), whereas patient samples comprised 0% wild genotype CC, approximately 82% mutant genotype CT, and 18% mutant genotype TT (Table 4). Furthermore, the comparison between male and female in both control and patient samples showed negligible frequency differentiation of indicated genotypes (Table 5). Taken together, these results demonstrate that the wild genotype CC within the position 589 in IL-4 is completely disappeared and modified to the mutant genotypes TT in samples from HCV infected patients.

Production of IL-4 is strongly interrupted in HCV patients

To determine whether the mutant genotypes IL-4-589/TT and IL-10-592/CC affected the expression profile of IL-4 and IL-10, quantitative reverse transcription (qRT-PCR) was used to detect the relative expression of indicated interleukins. The results demonstrated that the mRNA of IL-4 was strongly reduced in HCV infected patients; however, the expression of IL-10 showed normal levels compared with healthy individuals. As expected, IL-6 showed extremely high expression in HCV patients compared to control individuals (Figure 3A).

Figure 2. IL-4-589 C/T SNP in HCV patient and normal persons. **(A)** Control samples reveal the different genotype polymorphisms indicated by wild genotype CC, lanes 3 and 6, in addition to the mutant genotype TT, lanes 1, 2, 4, and 5. **(B)** Patient samples indicate the mutant genotype CT in the first 4 lanes in addition to the mutant genotypes TT in lanes 5 and 6. **(C)** The percentage of genotypes and alleles frequencies in both control and patient samples.

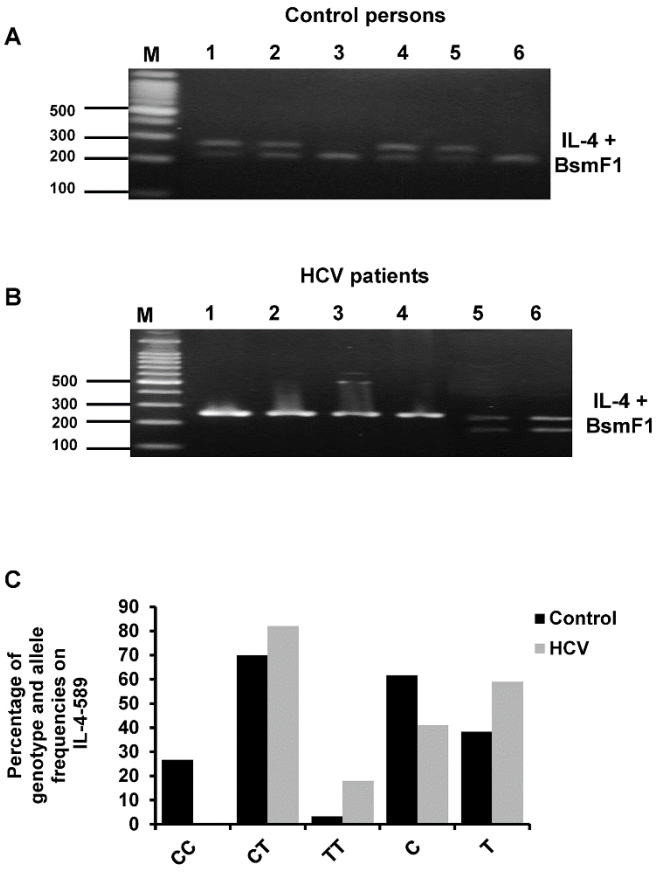


Figure 3. Expression profile of indicated interleukins in HCV patients. **(A)** Relative expression of IL-4, IL-6, and IL-10 at RNA level in samples derived from HCV patients in comparison with samples obtained from healthy individuals. Error bars indicate the standard deviation (SD) of tow impendent experiments. **(B)** Concentration of serum IL-4 indicated by pm/mL in HCV-infected patients compared to healthy individuals. Error bars indicate SD of tow impendent experiments. **(C)** Concentration of serum IL-6 indicated by pm/ml in patients compared to control samples. Error bars indicate SD of tow impendent experiments. **(D)** Concentration of serum IL-10 indicated by pm/mL in samples obtained from infected patients compared to control samples. Error bars indicate SD of tow impendent experiments (n = 8).

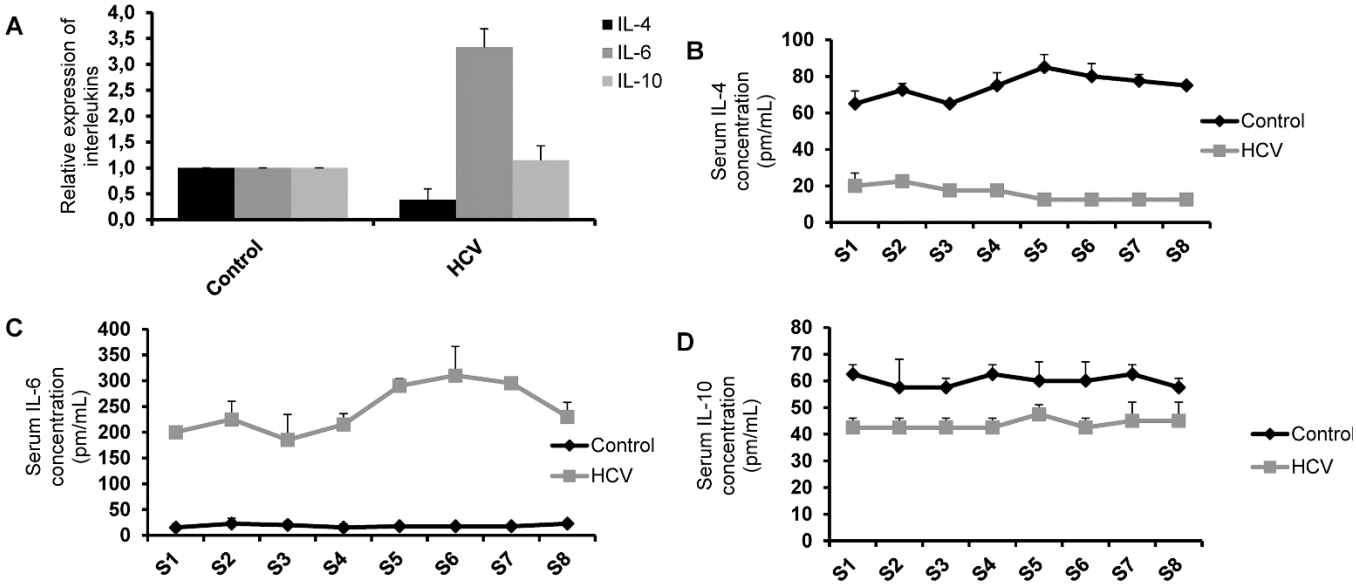


Table 3. Genotype frequencies of IL-10-592 assessed between males and females in control and patient samples.

IL-10-592	Control (n = 120)				P[x]	HCV (n = 100)				P[x]
	Wild type		Mutant			Wild type		Mutant		
	No	%	No	%		No	%	No	%	
Male	4	3.3	50	41.6	0.02*	4	4	38	38	0.03*
Female	4	3.3	62	51.6	0.01*	6	6	52	52	0.01*
P[x]		1.000		0.712			0.81		0.08	

Table 4. Polymorphism frequencies of IL-4-589 in control and patient samples.

IL-4-589	HCV patients (n = 100)		Control persons (n = 120)		P
	No	%	No	%	
CC	0	0.0	32	26.7	
CT	82	82.0	84	70.0	
TT	18	18.0	4	3.3	0.001**
C	82	41	148	61.7	
T	118	59	92	38.3	0.001**
Wild phenotype CC	0	0.0	32	26.7	
Mutant phenotype (CT and TT)	100	100.0	88	73.3	0.0320*

Table 5. Genotype frequencies of IL-4-589 assessed between males and females in control and patient samples.

IL-4-589	Control (n = 120)				P[x]	HCV (n = 100)				P[x]
	Wild type		Mutant			Wild type		Mutant		
	No	%	No	%		No	%	No	%	
Male	8	6.6	40	33	0.02*	-	-	42	42.0	0.001**
Female	24	20	48	40	0.06*	-	-	58	58.0	0.001**
P[x]		0.03*		0.5			0		0.4	

The expression of corresponding protein of IL-4 was also reduced, as demonstrated by a specific ELISA (Figure 3B). Additionally, production of IL-6 was dramatically increased while secretion of IL-10 showed negligible differentiation in HCV patients compared with healthy individuals (Figure 3C and D). Collectively, these data strongly suggest that the expression profile of IL-4 is inversely associated with HCV replication due to its mutant genotype IL-4-589/TT in infected patients.

Discussion

In this study, we found that the mutant genotype IL-10-592/CC was particularly increased in samples derived from HCV-infected patients compared with healthy individuals, without affecting the expression of IL-10. Of the subjects with persistent HCV infection, 10% were genotype IL-10-592/AA, 34% were mutant genotype IL-10-592/AC, and 56% were mutant genotypes and IL-10-592/CC. Control subjects showed 6.7% of genotype IL-10-592/AA, 50% of mutant genotype IL-10-592/AC and 43.3% of mutant genotypes and IL-10-592/CC. Interestingly, our findings indicate that the IL-4-589 SNP was associated with persistent HCV infection and modulated IL-4 expression. Samples with HCV infections had significantly lower IL-4-589/CC and higher IL-4-589/TT genotype frequency than the controls, accompanied by low expression levels of IL-4. A total of 26.7% of the subjects were IL-4-589/CC, while almost 70% of samples that obtained from HCV-infected patients were IL-4-589/TT.

IL-10 is a pleiotropic anti-inflammatory cytokine that is produced by various immune cells including monocytes, macrophages, B cells, T helper type 1 (Th1), T helper type 2 (Th2), and regular T cells (Tr). Secretion of IL-10 regulates the proliferation and differentiation of immune cells resulted in depletion of pro-inflammatory effects and inhibition of viral replication. Therefore, IL-10 has been therapeutically exploited for the treatment of various autoimmune diseases such as bowel and rheumatoid disorders and several infectious diseases such as HCV, human immunodeficiency virus (HIV), and influenza A virus infection [25-27]. Based on single nucleotide substitution, investigation of IL-10 gene polymorphism plays critical role in the biological studies of its regulatory functions that are associated with different disease and disorders. For instance, the potential genotypes within IL-10-592 are associated with histological activity index (HAI) but not with RNA levels of HCV patients [22]. Furthermore, IL-10-592/AC genotype is associated with abnormal serum level and severity of Hashimoto's disease [18,27]. Evidence indicated the possible connection of IL-10-819 genotype polymorphisms and the activity of hepatic inflammatory and fibrosis in patient infected with HCV genotype 3 [22]. In our study, the investigation of possible genotype polymorphisms indicated within IL-10-592 showed high levels of the mutant genotype CC in HCV patient and confirms the negative correlation between HCV RNA and IL-10 secretion. Our findings revealed that the association between IL-10-592 genotype polymorphisms and HCV-RNA level is not significant in Egyptian patients. Furthermore, the expression and secretion of serum IL-10 in HCV-infected subjects was interrupted compared to healthy control subjects, indicated by an ELISA test. This interruption of IL-10 secretion in patients could be due to the genotype of HCV common in Egypt. Notably, genotype 4a is the most common HCV variant in Egypt (78% of cases). HCV genotype 4 represents more than 17 million total global HCV infections and is prevalent in Africa, the Middle East, and some European countries [29,30]. The analysis of HCV genotype 4 in Egypt demonstrated that HCV genotype 4a is extremely variable from other genotypes in terms of sequences and immunological determinants that might alter virus-host interaction and cytokine production [31]. On the other hand, IL-4 is mainly produced by activated Th2 cells that also produce IL-5 and IL-13. IL-4 is a protein of 129 amino acids which is synthesized as a precursor with an hydrophobic secretory signal sequence of 24 amino acids. Production of IL-4 promotes the expression of major histocompatibility complex (MHC) class 2 antigen and stimulates the proliferation and differentiation of activated B cells [32]. Like IL-10, the frequency of IL-4/589 gene polymorphisms is associated with various disease and disorders such as asthma and allergies [33]. Connected with viral infection, the IL-4-589/CT genotype is associated with spontaneous HCV clearance, while the CC genotype is linked with abnormal serum ALT levels in HCV-infected patients [19,34]. Additionally, gene polymorphism of IL-4-589 is differentially influenced by the presence of other viruses such as HBV and HIV [19,35]. Here, the data revealed a positive association between HCV load of viremia and the mutant genotype IL-4-589/TT accompanied with inverse proportion with the wild genotype IL-4-589/AA and, subsequently, IL-4 production. Interestingly, the mutant genotype TT of IL-4 occurred in C-589T promoter locus in which the nucleotides CC have been modified to TT. This

modification in C to T nucleotide indicates the possible involvement of DNA methylation and epigenetic modification of IL-4 promoter sequences and, subsequently, disturbance of IL-4 expression in HCV-infected patients [24,36]. Our results indicate that high level of HCV load of viremia leads to high secretion of IL-6, which regulates interferon regulatory factor 1(IRF1) and inhibits cell growth, leading to stimulation of necrosis factors and cell death [7,37]. Collectively, these data confirm the association between HCV infection and the mutant genotypes IL-4-589/TT and IL-10-592/CC resulted in low production of IL-4 and IL-10.

Conclusions

In the current study, we investigated the possible interleukin SNPs that may be associated with HCV infection in Egyptian patients. A total of 100 samples were derived from patients infected with genotype 4a in addition to 120 samples obtained from healthy controls. SNPs of IL-10/592 and IL-4/589 were investigated, and their influence on corresponding gene expression was monitored. Our findings revealed a high level of the mutant genotype CC within IL-10/592 associated with HCV infection without affecting the expression of IL-10 in derived samples. Interestingly, the mutant genotype TT within IL-4/589 was highly increased in samples derived from infected patients, accompanied with low expression of IL-4. The mutant genotype IL-4/589-TT is induced in a specific location of gene promoter instead of IL-4/549-CC, indicating the possible involvement of methylation activity and epigenetic modification of IL-4 during HCV replication.

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