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The association of single nucleotide polymorphisms of Toll-like receptor 3, Toll-like receptor 7 and Toll-like receptor 8 genes with the susceptibility to HCV infection

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ABSTRACT

Background: Single nucleotide polymorphisms (SNPs) of Toll-like receptors (TLRs) are linked with functional modification of cytokine responses. In chronic hepatitis C virus (HCV) infection, studies of *TLR* polymorphisms have primarily targeted receptor pathways implicated in viral immune responses. We hypothesized that one or more variant(s) of *TLR3, TLR7* and *TLR8* are associated with different outcomes of HCV infection.

Materials & methods: A total of 3368 subjects from 850 families were recruited and divided into three main groups categorized as chronic HCV CHC spontaneous viral clearance (SVC), and controls. All individuals were genotyped for three SNPs for *TLR3*, two SNPs for *TLR7*, and two SNPs for *TLR8* using allelic discrimination real-time PCR.

Results: Carriage of the C allele in three SNPs of *TLR3* (rs3775290, rs3775291, and rs5743312), the C allele in *TLR7* (rs3853839) in females only, and the C allele in *TLR8* (rs3764879) in males only were significantly higher in SVC group than CHC group ($P < 0.001$), while carriage of the T allele in *TLR7* (rs179008) in females only and the A allele in *TLR8* (rs3764880) in both males and females were significantly higher in CHC infection more than SVC group ($P < 0.001$).

Conclusion: The C allele is protective of HCV in *TLR3, TLR7* (rs3853839) in females only, and *TLR8* (rs3764879) in males only, while risk of infection is linked to the T allele in *TLR7* (rs179008) in females only and the A allele in *TLR8* (rs3764880) in both sexes.

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Introduction

Hepatitis C virus (HCV) is a major health issue with over 185 million people estimated to be currently infected with HCV worldwide [1]. The yearly emergence of approximately 125,000 newly diagnosed viraemic cases in Egypt [2,3] does little to justify the high success rates of national programmes in controlling this infection [4]. The final outcome of HCV infection is ultimately determined by both innate and adaptive immune responses that lead to either resolution of the infection or progression to persistent infection with its array of associated complications [5,6].

Infection of host cells results in recognition of various pathogen-associated molecular patterns by the host as 'non-self'. Important factors in self/non-self are Toll-like receptors (TLRs), pattern recognition receptors that enhance specific immune responses that regulate expression of specific immune response genes [7]. In humans, 10 functional TLRs (designated TLR1 to TLR10) have been identified, all of which exhibit a conserved intracellular domain associated with an exclusive extracellular receptor that is specifically recognized for ligand binding. Binding of TLR3 (CD283, coded for by *TLR3* on chromosome 4q35.1) to its particular ligand results in stimulation of several signal pathways concluding in enhanced production of cytokines, chemokines, and various types of interferons, eventually generating an antiviral effect through inhibition of either protein synthesis or viral replication [8,9]. *TLR7* and *TLR8* are sex-linked by reason of their location on the X chromosome. These TLRs have the ability to recognize single-stranded viral RNAs [10], the attachment of which to endosomes generates type I and type III IFNs as well as pro-inflammatory mediators through activation of interferon regulatory factor 7 and nuclear factor kB respectively, depending on cell [11].

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Several single nucleotide polymorphisms (SNPs) of *TLRs* are linked with cytokine responses that are either modified or defective. Some *TLR* SNPs have been noted in diabetes mellitus, cardiovascular and renal diseases, as well as infections such as viral hepatitis [12]. Studies on the link of *TLR* polymorphisms and chronic HCV infection have focused on receptor pathways implicated in the immune response to the virus [13], including those of *TLR3, TLR7* and *TLR8* (latter two both sex-linked genes mapped to chromosome X) [11] with polymorphisms shown to link with either susceptibility to or progression of HCV infection [14]. However, conflicting results in different populations provide the basis for the unmet need for further studies to identify functional TLR polymorphisms and their relationship to progression of disease and response to therapy. We hypothesized that one or more variant(s) of *TLR3, TLR7* and *TLR8* are associated with different aspects of HCV infection.

Materials and methods

We tested our hypothesis on 3368 subjects during the period January 2013 to May 2017, who were studied in the Molecular Genetic Unit in Endemic Hepatogastroenterology and Infectious Diseases, Faculty of Medicine, Mansoura University. All subjects gave written informed consent and the approval of all local research ethics committees in each centre were obtained. Subjects were divided into three groups. The first was 1800 chronically HCV-infected patients (CHC) defined by anti-HCV antibodies and detectable HCV RNA. The second was 1460 healthy non-infected family members characterized by the absence of HCV antibodies and undetectable HCV RNA in two samples taken at least 6 months apart and without prior history of antiviral therapy. The third was 108 family members who comprised a spontaneous virus clearance group (SVC), defined as having detectable HCV antibodies and with HCV RNA level below the limit of detection in two consecutive samples taken at least 6 months apart in absence of prior history of any antiviral treatment.

Three ml blood samples were taken: one ml into k2EDTA for DNA analysis was aliquoted and stored at −50 °C, the remainder into plain tubes, allowed to clot, centrifuged at 4000 rpm for 10 min, the serum separated and aliquoted into 250 µl tubes to be stored at −50 °C. Anti-HCV antibodies were assessed by ELISA (Abbott Laboratories, Abbott Park, IL, USA), followed by quantitative PCR for patients found to be anti-HCV positive.

All individuals were genotyped for a total of seven SNPs: three in *TLR3* (rs3775290, rs3775291 and rs5743312), two in *TLR7* (rs3853839 and rs179008) and two in *TLR8* (rs3764880 and rs3764879). All samples were genotyped twice in two independent sessions. However, because some samples failed to amplify or/and discriminate, they were eventually excluded. As both *TLR7* and *TLR8* are sex-linked, they were further divided into male and females. Molecular diagnosis was carried out by first extraction and purification of genomic DNA from leucocytes of peripheral blood samples using a commercial Qiagen DNA isolation kit (Qiamp® DNA Minikit, Qiagen, Germany), according to the manufacturer's instructions. DNA was quantified by optical density at 260/280 nm (DNA Nanodrop, Thermoscientific, UK) to evaluate the purity and concentration. DNA quantification was done by 2% agarose gel electrophoresis with EtBr: 5 µl of each extracted DNA was mixed with 2 µl of the 2X loading dye and injected in separate lanes. In addition, 5 µl of 1 kb plus DNA Ladder (Tiagen Biotech, Beijing China) was injected into the first lane. The gel was run at 100 V for 30 min, then examined on the UV transilluminator to visualize the DNA bands.

The seven SNPs in *TLR 3, 7* and *8* were evaluated in all patients using real-time PCR (PCR Model 7500; Applied Biosystems, Foster City, CA, USA) incorporating ready-made labelled SNP probes utilizing fluorescein-amidite (Applied Biosystems). Allelic genotyping of each DNA sample allowed for detection of three SNPs in *TLR3* (rs3775291, rs3775290, and rs5743312), two in *TLR7* (rs3853839 and rs179008) and two in *TLR8* (rs3764880 and rs3764879). Also purchased were TaqMan® Universal Master Mix II (2×) (Applied Biosystems), DNA template, RNase-free water, and optical plate (MicroAmp® Optical 96-Well Reaction Plate, Applied Biosystems).

Allele discrimination was performed using a TaqMan®SNP Genotyping assay kit on real-time PCR 7500 ABI with software version: 2.0.1, (Applied Biosystem). Endpoint reads on the real-time PCR instrument were subsequently plotted by the analysis software that makes allowances for auto-calling genotypes, thereby minimizing manual intervention. Plotting is done generally by allele 1 (VIC® dye) on the X-axis and allele 2 (FAM™ dye) on the Y-axis (Figure 1) (https://tools.thermofisher.com/content/sfs/manuals/TaqMan_SNP_Genotyping_Assays_man.pdf). After each run, the genotyped data was reviewed by two independent examiners who were blind to the all subject status of the samples. The genotyped data was analysed using SPSS software program, Version 21.0. Qualitative variables were compared using Chi-square and Fisher's exact tests. Each group was tested separately for Hardy-Weinberg equilibrium using x^2 test. Allele carriage corresponds to the number of people carrying at least a single copy of a particular allele, while allelic frequency equates the number of appearances of specific test allele divided the overall count of alleles in the group.

The odds ratio (OR) and 95% confidence interval (CI) of carriage of a specific allele were computed and examined in contrast to absence of carriage of the target allele (Med Calc Statistical Software Version 16.4.3; Med Calc

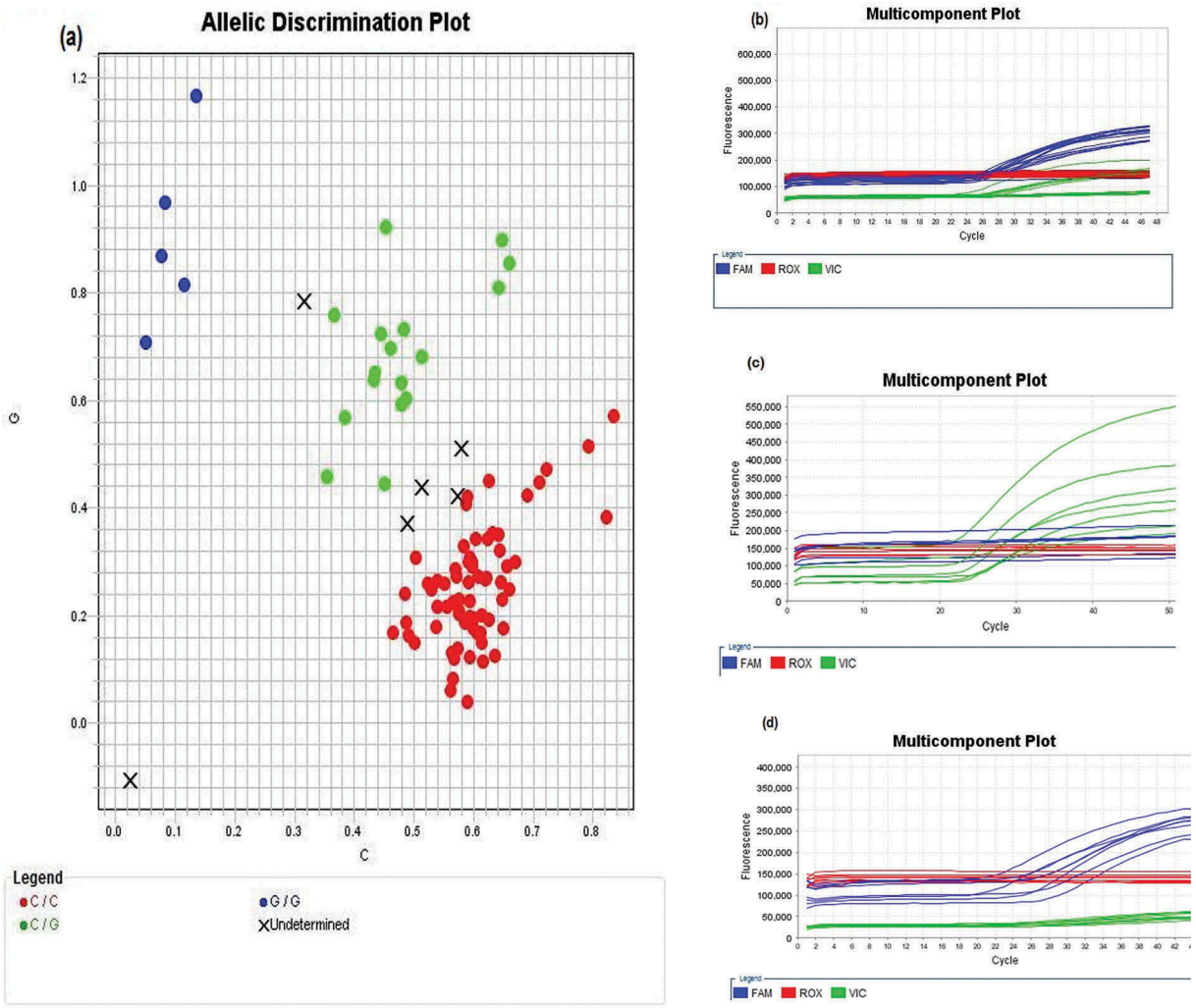

Figure 1. The output file of allele discrimination software plot and multicomponent plot of TLR7 rs3853839. (a): Allelic discrimination plot: 1 represents the sample with FAM homozygote genotyping, 3 represents the sample with VIC and FAM heterozygote genotyping, 2 represents the sample with VIC homozygote genotyping and X represents the undetermined samples. (b): The CG genotyping subjects (carrying the C and G allele) (heterozygote) among women. (c): The CC genotyping subjects (carrying the C allele only) (homozygote). (d): The GG genotyping subjects (carrying the G allele only) (homozygote).

(Software bvba, Ostend, Belgium)). Values of $P < 0.05$ were considered significant. Use of numerous dependent or independent statistical tests synchronously on a set of data required adjustment of the *P* value to be Bonferroni-corrected (*Pc*) [15].

Results

Demographics of the groups were as follows: the 1800 CHC patients comprised 954 males and 846 females of mean age (standard deviation, SD) 40.5 (9.5) years. The 1460 healthy control subjects were 876 males and 584 females aged 44.7 (10.9 years). The 108 subjects with SVC were 43 males and 65 females, aged 43.0 (10.5) years (age and sex difference both $P < 0.01$)

Table 1 shows allele carriage of *TLR3, TLR7* and *TLR8* SNPs among the groups. All were in Hardy-Weinberg equilibrium, except the males with regards to *TLR7* and *TLR8*. Table 2 shows associations of the *TLR* SNPs. The C allele of rs3775290 (C/T), rs3775291 (C/T) and rs5743312 (C/T) TLR3 polymorphisms were significantly higher in SVC compared to both CHC and control groups ($P < 0.001$), while no significant difference was detected between CHC group and the control group (*P* value not applicable). The central section of Table 2 shows SNPs of the sex-linked *TLR7* among the groups. The carriage of the C allele of rs3853839 (C/G) was significantly higher in SVC group compared to CHC in females ($P < 0.001$) but not in males (*P* value is not applicable). Figure 1(a-d) represent the output file of allele discrimination software plot and multicomponent plot of *TLR7* rs3853839. Carriage of the T allele of *TLR7* rs179008 (A/T) was higher in CHC group in comparison to SVC group in females ($P < 0.001$) but not in males ($P > 0.05$).

The lower section of Table 2 describes the genotype frequencies of SNPs of sex-linked *TLR8* and reveals that the A allele of rs3764880 (A/G) in both sexes is a significant risk allele, being higher in CHC

Table 1. Allele carriage of TLR SNPs among different groups.

Gene	SNP		Groups	Genotyping N(ratio) Wild	Het	Rare	*P* value
TLR3	**rs3775290**		**Control (1337)**	852 (63.7%)	417 (31.2%)	68 (5.1%)	0.07
			CHC (1308)	525 (50.1%)	602 (46.0%)	181 (3.9%)	0.69
			SVC (108)	63 (58.3%)	42 (38.9%)	3 (2.8%)	0.19
	rs3775291		**Control (1328)**	756 (56.9%)	496 (37.4%)	76 (5.7%)	0.65
			CHC (1284)	488 (38.0%)	580 (45.2%)	216 (16.8)%	0.05
			SVC (108)	52 (48.2%)	48 (44.4%)	8 (7.4%)	0.49
	rs5743312		**Control (1332)**	852 (64.0%)	412 (30.9%)	68 (5.1%)	0.05
			CHC (1292)	516 (40.0%)	600 (46.4%)	176 (13.6%)	0.006
			SVC (108)	55 (50.9%)	49 (45.4%)	4 (3.7%)	0.08
TLR7	**rs3853839**	**Male**	**Control (705)**	505 (71.6%)	0	200 (28.4%)	NA
			CHC (785)	415 (52.9%)	0	370 (47.1%)	NA
			SVC (43)	25 (58.1%)	0	18 (41.9%)	NA
		Female	**Control (299)**	153 (51.1%)	112 (37.5%)	34 (11.4%)	0.06
			CHC (263)	66 (25.1%)	116 (44.1%)	81 (30.8%)	0.06
			SVC (65)	38 (58.4%)	20 (30.8%)	7 (10.8%)	0.10
	rs179008	**Male**	**Control (680)**	495 (72.8%)	0	185 (27.2%)	NA
			CHC (740)	445 (60.1%)	0	295 (39.9%)	NA
			SVC (43)	29 (67.4%)	0	14 (32.5%)	NA
		Female	**Control (139)**	84 (60.4%)	46 (33.1%)	9 (6.5%)	0.43
			CHC (124)	41 (33.1%)	54 (43.5%)	29 (23.4%)	0.18
			SVC (65)	43 (66.2%)	19 (29.2%)	3 (4.6%)	0.08
TLR8	**rs3764880**	**Male**	**Control (654)**	378 (57.8%)	0	276 (42.2%)	NA
			CHC (864)	360 (41.7%)	0	504 (58.3%)	NA
			SVC (43)	31 (72.1%)	0	12 (27.9%)	NA
		Female	**Control (680)**	210 (30.9%)	340 (50.0%)	40 (6.0%)	0.71
			CHC (619)	143 (23.1%)	285 (46.0%)	191 (30.9%)	0.09
			SVC (65)	41 (63.1%)	19 (29.2%)	5 (7.7%)	0.21
	rs3764879	**Male**	**Control (562)**	361 (64.2%)	0	201 (35.8%)	NA
			CHC (542)	245 (45.2%)	0	297 (54.79%)	NA
			SVC (43)	30 (69.8%)	0	13 (30.2%)	NA
		Female	**Control (239)**	96 (40.2%)	121 (50.6%)	22 (9.2%)	0.06
			CHC (208)	80 (38.5%)	97 (46.7%)	31 (14.9%)	0.86
			SVC (65)	47 (72.3%)	15 (23.1%)	3 (4.6%)	0.23

SNP: Single nucleotide polymorphism; SVC: spontaneous viral clearance; CHC: chronic hepatitis C; HET: Heterozygosity; TLR = Toll-like receptors.

group when compared to both SVC ($P < 0.001$) and control groups ($P < 0.001$), but no significant difference was detected when SVC group was compared to healthy control group ($P > 0.05$). The C allele of TLR8 rs3764879 (C/G) was a significant protective allele in males ($P < 0.001$) but not in females ($Pc > 0.05$).

Discussion

TLRs play a major role in certain responses of the innate immune system. Variation in *TLRs* has been linked to vaccination efficiency and susceptibility to various diseases, such as diabetes mellitus, cardiovascular and renal diseases, rheumatoid arthritis, sepsis and malignancies, non-alcoholic fatty liver disease, alcoholic liver disease, viral hepatitis, autoimmune liver disease, hepatic fibrosis and liver neoplasms, although some links are unclear [16–23]. TLR3 is expressed intracellularly, and recognises double-stranded RNA with subsequent antiviral and anti-tumour effects, while both TLR7 and TLR8 recognise single-stranded RNA and elicit an immune reaction that may be associated with impaired or altered cytokine response ultimately accounting for disease susceptibility and progression of HCV infection [11,13,24–26].

Our hypothesis was that any of seven *TLR* SNPs are linked with susceptibility to chronic HCV infection, or to spontaneous clearance of the virus (the latter implied by the presence of anti-HCV antibodies but lack of circulating viral nucleic acid). We report that the C allele of the three *TLR3* SNPs (rs3775290, rs3775291 and rs5743312) was significantly higher in SVC compared to CHC infection, but no significant association was detected between negative controls and SVC, indicating that this SNP is strongly linked with protection against development of chronic HCV infection. Askar et al showed a lack of association between *TLR3* promoter genotype and *TLR3* mRNA expression, where *TLR3* expression seemed to be higher in TT homozygotes than in C allele carriers [27], but the susceptibility to HCV infection was not investigated in that study. A meta-analysis on *TLR3* associations found TT/CT genotypes to be linked with a higher liability of HCV infection, but that the C allele was protective [28]. However, another study of *TLR3* rs3775291 showed no polymorphism link with HCV infection susceptibility, whilst an earlier study found that there was no significant difference between HCV-positive patients and controls with regards to the C allele of *TLR3* rs3775290 but that the T-allele linked with late stage hepatic fibrosis [29]. Concerning other

Table 2. Association of SNPs of minor alleles in TLR 3 SNP_s among different groups.

Gene	SNP	Polymorphism	Tested Allele		Groups	OR (95% CI)	*P* value	Bonferroni-corrected *P value* (*Pc*)
TLR3	**rs3775290**	**C/T**	**C**		**CHC vs. Control**	0.3 (0.2 – 0.4)	< 0.0001	< 0.0001
					CHC vs. SVC	0.2 (0.1 – 0.5)	0.004	0.01
					SVC vs. Control	0.5 (0.2 – 1.7)	0.3	NA
	rs3775291	**C/T**	**C**		**CHC vs. Control**	0.31 (0.2 – 0.4)	< 0.0001	< 0.0001
					CHC vs. SVC	0.4 (0.2 – 0.8)	0.01	0.04
					SVC vs. Control	1.3 (0.6 – 2.8)	0.5	NA
	rs5743312	**C/T**	**C**		**CHC vs. Control**	0.3 (0.3 – 0.5)	< 0.0001	< 0.0001
					CHC vs. SVC	0.2 (0.1 – 0.7)	0.01	0.02
					SVC vs. Control	0.7 (0.3 – 2)	0.5	NA
TLR7	**rs3853839**	**C/G**	**C**	**Male**	**CHC vs Control**	0.4 (0.4 – 0.6)	< 0.0001	< 0.0001
					CHC vs SVC	0.8 (0.4 – 1.5)	0.5	NA
					SVC vs Control	1.8 (1.0 – 3.4)	0.1	0.2
				Female	**CHC vs Control**	0.3 (0.2 – 0.5)	< 0.0001	< 0.0001
					CHC vs SVC	0.3 (0.1 – 0.6)	0.002	0.01
					SVC vs Control	0.9 (0.4 – 2.2)	0.9	NA
	rs179008	**A/T**	**T**	**Male**	**CHC vs Control**	1.8 (1.4 – 2.2)	< 0.0001	< 0.0001
					CHC vs SVC	2.0 (1.0 – 4.1)	0.04	0.1
					SVC vs Control	1.2 (0.6 – 2.4)	0.7	NA
				Female	**CHC vs Control**	3.1 (1.9 – 5.1)	< 0.0001	< 0.0001
					CHC vs SVC	4.0 (2.1 – 7.5)	< 0.0001	< 0.0001
					SVC vs Control	1.3 (0.6 – 2.4)	0.4	NA
TLR8	**rs3764880**	**A/G**	**A**	**Male**	**CHC vs. Control**	1.9 (1.6 – 2.4)	0.0001	0.0003
					CHC vs. SVC	3.6 (1.8 – 7.1)	0.0002	0.001
					SVC vs. Control	1.9 (1.0 – 3.7)	0.07	0.2
				Female	**CHC vs. Control**	1.5 (1.2 – 1.9)	0.002	0.01
					CHC vs. SVC	5.7 (3.3 – 9.8)	< 0.0001	< 0.0001
					SVC vs. Control	3.8 (2.3 – 6.5)	< 0.0001	< 0.0001
	rs3764879	**C/G**	**C**	**Male**	**CHC vs. Control**	0.5 (0.4 – 0.6)	0.0001	0.0003
					CHC vs. SVC	0.4 (0.2 – 0.6)	0.0002	0.001
					SVC vs. Control	0.8 (0.5 – 1.4)	0.4	1.3
				Female	**CHC vs. Control**	0.3 (0.1 – 0.6)	0.002	0.005
					CHC vs. SVC	0.3 (0.1 – 0.9)	0.04	0.1
					SVC vs. Control	0.5 (0.1 – 1.6)	0.2	0.7

SNPs, others reported no association between both alleles of the *TLR3* rs5743312 SNP with HCV infection [30]. However, these studies are limited by relatively small sample size and lack of cases with spontaneous viral clearance.

The G allele of the *TLR7* SNP rs3853839 was significantly higher in SVC compared to CHC infection in females only, indicating that the G allele is more protective than in males. Conversely, the A allele of *TLR7* SNP rs179008 was significantly higher in CHC infection compared to SVC in males only, indicating that the A allele affords more risk compared to females, possibly attributed to sex hormonal impact on the innate and adaptive immune responses of the infected host [18,31]. Plasmacytoid dendritic cell responses mediated by TLRs were shown to be positively regulated by oestradiol cell-intrinsic oestrogen receptor signalling which may stimulate IFN-α release with subsequent elimination of HCV infection [25].

Askar et al reported that T allele (risk allele) increases susceptibility to chronic HCV infection in both sexes in addition to reducing the capability of spontaneous clearance of the virus. In *TLR7*, encoding of the leucine (Leu) variant by the T allele of SNP rs179008 results in substitution of the wild allele A-encoded glutamine (Gln) at codon 11 in the protein (Gln11Leu), is associated with higher risk of infection with HCV and less likelihood of adequate response to IFN-α-based therapy in chronic HCV-infected females [14].

Contrary to our results, Yue et al found a link between the C allele of rs3853839 and haplotype GC and CC in females included in the HCV natural clearance group more than those of the HCV persistence group, attesting to the protective nature of the C allele [32]. Furthermore, Fakhir et al. reported both males and females carrying the A allele of *TLR7* rs179008 were associated with spontaneous viral clearance [33]. We found the A allele of *TLR8* rs3764880 is a risk allele for the

development of chronic HCV in both sexes, while the C allele of rs3764879 is a protective allele against development of chronic HCV in both subpopulations. This extends a Moroccan study where the G allele of *TLR8* rs3764880 was associated with spontaneous clearance of HCV in both sexes while the C allele of *TLR8* rs3764879 and the A allele of TLR8 rs3764880 were significantly associated with progression of liver disease in males [33].

Wang et al. reported that, in a Taiwanese population, *TLR8-129* C (rs3764879) allele SNPs were significantly more frequent in males with chronic HCV infection when compared with healthy controls while no link was found in females [34]. However, when comparing allele frequencies between different racial groups, the Taiwanese had frequencies different to those of Caucasians and Nigerians, possibly explaining the differences with our results. However, the relatively small sample size and the absence of individuals with evident spontaneous viral clearance to be compared with patients with chronic HCV in that study [34], instead of healthy controls, makes comparisons difficult. Alterations in *TLR8* may affect responses of the immune system to infection with HCV leading to decreased IFN-α release, resulting in lower levels of immune activation and possibly explaining the increased susceptibility to chronic HCV infection associated with these mutations [35]. However, the exact effect of *TLR8* polymorphisms on HCV infection remains to be established.

We recognize a weakness in our data is the relatively small number of subjects proven to have spontaneously cleared an HCV infection, and that this group has a larger proportion of females. Nevertheless, this work represents an advance in biomedical science because it shows that variations in certain *TLR* polymorphisms are linked with the outcomes of HCV infection, these being viral clearance or persistence to chronic disease.

Summary table

What is known about this subject

- The final outcome of HCV infection is either resolution (20%) or development of persistent infection (80%).
- *TLR3* and sex-linked *TLR7* and *TLR8* enhance specific immune responses.
- Studies examining the association of *TLR* polymorphisms with the susceptibility to HCV infection report conflicting results in different ethnic populations.

What this paper adds:

- The C allele is a protective allele (linked to spontaneous clearance of HCV) in *TLR3, TLR7* (rs3853839) in females only and *TLR8* (rs3764879) in males only.
- Risk alleles (promoting development of chronic HCV) are the T allele of *TLR7* (rs179008) in females only and the A allele of *TLR8* (rs3764880) in both sexes.

Disclosure statement

The authors declare no conflict of interests related to this paper.

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