CROSS-SECTIONAL STUDY OF IL28B POLYMORPHISMS AMONGST SAUDI ARABIAN AND BRITISH HCV PATIENTS

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Abstract

Several studies published in 2009 suggested that single nucleotide polymorphisms (SNPs) and viral markers in the IL28B gene locus (Ge et al., 2009; Suppiah et al., 2009 and Tanaka et al., 2009) are associated with increased risk of poor response to therapy. IL28B is a member of the interferon (IFN) cytokine family known as IFN III or *Lamda* (IFN-λ), which

includes three types: IFN-λ1 (IL-29), -λ2 (IL-28A) and -λ3 (IL-28B). These are encoded by 3 different genes located on chromosome 19 (Kotenko et al., 2003; Sheppard et al., 2003). rs12979860 genotype (CC, TT, C/T) was the first polymorphism of the IL28B gene to be identified and was associated with a twofold improvement with treatment of HCV infection amongst people of European (P= 1.063 X10^{-25}), Hispanic (P= 4.39 X 10^{-3}) and African-American (P= 2.06 X 10^{-3}) ancestry (Goldstein et al., 2009). Seventy per cent of Caucasians, 40% of African-Americans and 95% of Asians carry at least one copy of the rs12979860C variant allele (Hézode and Chevaliez, 2010). Amongst Egyptians, the CC genotype was significant (p<0.001) for 87.2% in SVR, CT for 25.5% but TT for only 10% and was associated with failure to respond to therapy (Hendy et al., 2011). The response rate was found to be higher amongst the CC and CT patients than the TT patients after 4 weeks of treatment. However, by week 8, viral clearance was more common amongst the CC and CT patients, although the CT rate of clearance was found to be closer to that of the TT than CC patients (Afendy et al., 2011; Chung et al., 2010; Hézode and Chevaliez, 2010; Goldstein et al., 2009). The second polymorphism (rs8099917-genotype (GG, TT, G/T)) was found in individuals of Australian and northern European ancestry. The GG genotype was strongly associated with a null virological response (NVR) to treatment with peg-IFN-α +RBV, whereas patients with the G/T or TT genotypes showed an increased probability of achieving an SVR (Thol et al., 2010; Toyoda et al., 2011). The GT genotype frequency was 42%, and the GG genotype frequency was 16.7% in the responding Egyptian patients (Hendy et al., 2011). Recent data have shown that the SNPs near the IL28B gene are not only associated with an SVR or non-response to treatment with peg-IFN-α + RBV but are also associated with a response to telaprevir-containing regimens (Rallon et al., 2010; Rauch et al., 2010; Akuta et al., 2010). The effects of the IL28B polymorphism on the kinetics of HCV clearance after therapy have also been investigated. Bochud showed that polymorphisms in IL28B were significantly correlated with the first phase of viral decline during peg-IFN-α + RBV therapy of chronic HCV infection, irrespective of HCV genotype (Bochud et al., 2011).

Aims - The aims of this study were to investigate the prevalence of IL28B polymorphisms in Saudi Arabian patients infected with hepatitis C virus (HCV), hepatitis B (HBV) and human immunodeficiency virus (HIV) and to compare the prevalence amongst the Saudi Arabian HCV patients with that amongst HCV-infected patients from the United Kingdom (UK). The correlation of the IL28B genotype with responsiveness to therapy was also evaluated.

Methods - This study included 328 patients who received therapy and whose demographic data were collected. The biochemical and virologic parameters were also defined to indicate their response to therapy.

An assay to examine the IL28b polymorphism in the human genome was performed, and the rs12979860C/T and rs8099917G/T variants were determined with a real-time PCR platform using an allelic discrimination method and a melting point with dynamic detection of the signals for both variants shown by fluorescent dyes during *in vitro* amplification. DNA from serum was extracted using the QIA gene (QIAxtractor) and amplified using the rt-PCR Light Cycler 480 supplied by Roche Diagnostic Company.

Results - This study illustrated that both of the polymorphisms (RS60 and RS17) interfered with the host response to treatment. The study first identified the HCV patients who achieved SVR and became responders (R). Amongst the patients who achieved SVR, the CC genotype was identified in 19% of them, the CT genotype in 22% and the TT genotype in 38.4 %. A significant correlation between RS-60 and the level of response was observed ($p < 0.001$). The results of the present study suggest that the non-responding patients (NR) had genotype frequencies for CT of approximately 53%, CC of approximately 50% and TT of 23%. NR and the IL28B (RS-60) genotypes were significantly correlated ($p < 0.001$), whereas amongst the partially responding patients (PR), similar patterns of frequency for genotypes CC and TT (7.7%) were observed more frequently than for CT (3.2%). RS-60 and partial response was significantly correlated (0.026). Transitional response (TR) patients showed a higher frequency of the TT genotype (23.1%) than the CT genotype (8.1%). However, the CC genotype frequency was the lowest (7.7%). The study also demonstrated that the predominate genotype for the HCV virus in the patients from the Kingdom of Saudi Arabia was 4 (87%). The frequency of RS-17 did not significantly correlate with treatment response ($P > 0.05$). The frequency of RS-17 and RS-60 in the UK samples was not significantly correlated with treatment response ($P > 0.05$).

In addition, in the NR, the HBV frequency of RS-17 GT was 25%, TT 66.3% and GG 0%. In patients who achieved SVR, approximately 47% of the genotypes were GT and 100% were GG; no TT genotypes were identified. The PR group showed a higher frequency for TT (33.3%) than GT (25%); no GG genotypes were identified. The RS-17 ($P > 0.05$ for all) and RS=60 ($P > 0.05$) genotype frequencies were not significantly correlated with treatment response.

In HIV patients, RS-17 and NR were significantly correlated ($P= 0.010$). The GG genotype was present in 53%, GT in 57.4% and TT in 22.2% of the patients who did not achieve SVR.

In the patients who achieved SVR, TT and GG had a similar frequency (26.3%) and GT had a frequency of 20.4%. In the PR group, TT was the most common genotype (37%), followed by GT (18.5%) and GG (10.5%). No significant correlations were observed between RS-17 and either PR or R (P > 0.05).

Treatment response and the frequency of IL28B for the RS-60 genotypes were significantly correlated (P= 0.001). Twenty-one per cent of the patients achieved SVR, with the highest frequency having the CT genotype (26%), followed by CC (11.8%). Forty-seven per cent of the HIV patients failed to respond. The CC genotype was the most common (77%), followed by TT (50%) and CT (40%). The relationship between IL28B for the RS60 genotypes and the NR group was statistically significant (P = 0.023). In the PR group, the most common genotype was TT (40%), followed by CT (23.3%) and CC (5.9%). The relationship between the IL28B RS60 genotypes and PR was statistically significant (P = 0.002). The results for the RS-17 genotype in KSA-HIV correlated significantly with viral load (P = 0.034).

Keywords : HCV, HIV, HBV, IL28B (RS-60, RS-17) genotypes, viral load

Introduction

Several studies have identified viral markers that are associated with a better SVR in patients receiving peg-IFN-α and RBV. In 2009, genome wide association studies (GWAS) revealed that single nucleotide polymorphisms (SNPs) in the IL28B gene locus, known also as type III IFN (IFNλ3), were associated with the control of HCV replication during acute infection and response to therapy. Ge, in the same year, identified a SNP (rs12979860) located in chromosome 19.3kb. Several GWAS publications have indicated that the genetic polymorphisms near the IL28B gene on chromosome 19 were associated with improved response to treatment, with a direct correlation to SVR in chronic HCV-infected patients (Ge et al., 2009).

This gene was then classified in the interferon (IFN) cytokine family and was known as IFN III or *Lamda* (IFN-λ) with three types: IFN-λ1 (IL-29), -λ2 (IL-28A) and -λ3 (IL-28B). These types were encoded by 3 different genes located on chromosome 19 (Kotenko et al., 2003; Sheppard et al., 2003). At the amino acid level, IFN-λ2 and -λ3 are closely similar, having a 96% sequence identity, whereas IFN-λ1 shares approximately 81% sequence identity with IFN-λ2 and IFN-λ3. The sequence of IFN-λ3 was shown to have two polymorphisms, G and C, at the start codon upstream of the 37 transition nucleotides. The two polymorphism residues are located at the AB loop of the IFN-λ structure in a variable

position flanked by the three isoforms (S in λ1, R in λ2 and K in λ3). A study by Thomas in 2008/09 determined that these polymorphisms are involved in the response of HCV to therapy (Thomas et al., 2008 and 2009).

HCV-infected hepatocytes can induce the expression of IFN-α/β and IFN-λ genes, which leads to the phosphorylation of STAT1 and STAT2, thereby forming STAT1-STAT2 heterodimers. The dimers then bind to IRF9 and form the ISGF3 complex, which then migrates to the nucleus to bind to the ISRE elements to facilitate the transcription of ISGs.

The binding receptors of IFN-λ can form the complex that is needed to activate JAK1 and TYK2. The complex also includes an intracellular domain of 270 aa (Hamming et al., 2010). The two kinases of the IFN-λ cross-phosphorylate to activate each other for the phosphorylation of the three tyrosine residues on the intracellular part of IFNλ- R1: Tyr343, Tyr406 and Tyr517. Tyr343 and Tyr517 then create a docking site for the Src Homology 2 (SH2) domain of the transcription factor STAT2 (Hamming et al., 2010). When STAT binds to IFN-λR1, it activates JAK1 and TYK2 and allows for the phosphorylation to transfer the tyrosine residue towards the C-terminal end of the STAT proteins. This docking site then serves as the SH2 domain. The STATs 1 and 2 activation that allows for the joining of IRF9 to form the ISGF3 complex is considered the main gate for IFN-λ activation, which in turn activates the STATs 3 and 5 (Kelly et al., 2010). To bind to the gamma activated sequence (GAS) and induce expression of the gene, the ISGF3 complex induces the transcription of the interferon-stimulated genes (ISGs) by its translocation into the nucleus and its interaction with a specific DNA sequence designated the IFN-stimulated response element (ISRE) (Dumoutier et al., 2004).

IFN-α/β and IFN-λ were reported to activate the MAP kinase pathway through JAK and p38 phosphorylation, as previously mentioned. IFN III can also raise the levels of MHC classes I and II and the chemokine receptor CCR7 to stimulate the migration of DCs to the lymph nodes and the spleen, thereby inducing immunity and demonstrating antiviral effects (Walter et al., 2004; Lasfar et al., 2006; Ank et al., 2006). It is anti-proliferative and acts with type I IFN in the immunomodulation of the Th1/Th2 balance in the immune response (Bartlett et al., 2005; Ank et al., 2008; Dellgren et al., 2009; Hartmann et al., 2010; Lasfar and Cohen-Solal, 2010).

The first polymorphism identified was the rs12979860-genotype (CC, TT, C/T), which was found to be associated with SVR with a twofold improvement in response to treatment amongst people of European (P= 1.063×10^{-25}), Hispanic (P= 4.39×10^{-3}) and African–American (P= 2.06×10^{-3}) ancestry. This occurred when the CC genotype was

present and in patients who did not achieve RVR (Goldstein et al., 2009). Approximately 70% of Caucasians, 40% of African-Americans and 95% of Asians carry at least one copy of the rs12979860C variant allele (Hézode and Chevaliez, 2010). The frequency of the CC genotype differs according to ethnic group and is also present at a much higher frequency in patients with European ancestry compared with those with African-American ancestry. The SNP, which is near the IL28B gene, is also a predictor of response to HCV treatment with IFN and RBV. After 4 weeks of treatment, the response rate was higher in CC and CT patients than in TT patients. However, by week 8, viral clearance was greater in CC and CT patients; the CT clearance rate was closer to that of the TT rather than that of the CC (Afendy et al., 2011; Chung et al., 2010; Hézode and Chevaliez, 2010; Goldstein et al., 2009).

The second polymorphism rs8099917 genotype (GG, TT, G/T) was found to be located at 8.9Kb from the end of the transcription of IL28B. A GWAS reported this SNP amongst Australians of northern European ancestry. Patients with the GG genotype showed a strong association with a null virological response (NVR) to treatment with peg-IFN + RBV, whereas patients with the G/T or TT genotypes showed an association with SVR (Thiol et al., 2010; Toyoda et al., 2011).

In addition, recent data (Rallon, 2010; Rauch, 2010; Akuta, 2010) have shown that not only are these SNPs near the IL28B gene closely associated with SVR or non-response to treatment with peg-IFN-α + RBV alone, but they are also associated with a response to Telaprevir-containing regimens.

The effects of IL28B polymorphism on the kinetics of HCV clearance after therapy have also been investigated. Bochud discovered that polymorphisms in IL28B were significantly correlated with the first phase of viral decline during peg-IFN-α + RBV therapy of chronic HCV infection, irrespective of HCV genotype (Bochud et al., 2011).

The present study used many samples from Saudi patients who were chronically infected with HCV, HBV and HIV, and compared them with samples and data output from HCV-infected patients in the United Kingdom. All samples were collected to evaluate the distribution of IL28B SNPs in the context of the predominant genotype in Saudi Arabia and the correlation with a virological response (VR). To our knowledge, such a study has not been previously performed on a Saudi population, and, therefore, the results should provide a broad picture of the influence of the predominant HCV genotype in the KSA and the alleles that correlate with response to treatment. A small number of patients from the UK were also used for comparison of IL28B polymorphisms. Viral load, therapeutic response and all other parameters were analysed for influence of the IL28B gene on the therapeutic response.

Materials and methods

IL28b polymorphism on the human genome

A real-time PCR platform using an allelic discrimination method was used to detect the rs12979860C/T and rs8099917G/T variants. The melting point with dynamic detection of the signals for both variants was given by fluorescent dyes during the *in vitro* amplification (PCR reaction). The probes were designed and authorised by Drs. Clewley and Foster to have a -10° Celsius higher melting temperature than the PCR primes and thus bind to the target before the primer. During the reaction and specifically during the polymerisation, the dyes were released; hence, the signals were released and measured at the end of each PCR cycle.

Samples and Patients

Samples were randomly collected from 315 patients at the Ministry of Health (MOH), King Saud Complex in Riyadh, Kingdom of Saudi Arabia. Demographical data and biochemical and virologic parameters were collected to indicate the patients' response to therapy.

One hundred fourteen patients chronically infected with the hepatitis C virus who were to be treated either with IFN-α or peg-IFN-α + RBV were categorised and divided into the following groups: responder (R), non-responder (NR), partial responder (PR), on treatment (OT) and stopping treatment (ST).

Another 101 samples from HBV-positive patients were collected, and samples from 100 HIV patients were also collected from in- and out-patients.

Baseline samples from 13 UK patients who were identified to have HCV genotype 1b were also subjected to IL28B examination.

A positive control was sent from RFH as a diluent of a plasmid and the IL28B SNPs in 100 µM.

Extraction

All samples were extracted using QIAxtractor. The guideline protocols were found at www.qiagen.com/goto/QIAxtractor.

Preparation
a- Reagents

1- Binding mixture: 1.14 g of Digest Enzyme (DX) was added to 100 ml of the binding reagent (DXB) and stored in a dark bottle at 2° - 8° C.

2- Digestion mixtures: in a 15-ml tube 1 part DX was diluted by 9 parts Liquid Digest Reagent (DXL), then mixed gently by inverting the tube 10 times. The DX was added to the DXL immediately before use, and then the sample was set on B1 on the QIAxtractor

worktable to be added to the DX digestion mixture within 10 minutes of adding the DX to avoid enzyme degradation.

b- Samples

Serum (150 µl) was resuspended in 50 µl PBS and stored at 2° - 8° C for up to 1 day before processing for the extraction. The frozen samples were then thawed and centrifuged. 200 µl was added to the plate and placed in position on the QIAxtractor worktable. The reagents were then put in place, and 10 ml of the Liquid Digest Reagent, without the Liquid Digest (DX), was transferred to the reagents. The DXL and DX were then immediately poured into each well before the run started. After completing the run, the elution plate was covered with the lid and removed from the elution chamber. The purified DNA was then stored at 2° - 8°C for use the next day.

Real-time PCR reaction
a- Programme

The rt-PCR Light Cycler 480 supplied by Roche was used for this study. The programme was set for the reaction of the two SNPs with the details of the programmes being provided by Drs. Clewley and Foster. For rs12979860C/T, the thermal cycler was set as follows: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 65°C for 1 min. The melting temperatures (TM) were adjusted by adding 5 more cycles at 72°C for 30 sec.

For rs809917G/T, the thermal cycler was set as follows: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Melting temperatures (TM) were increased by an additional 5 cycles at 72°C for 30 sec.

b- Reagent

The 2X master mix (MM) for 200 reactions in 50 µl of Taq DNA polymerase, dNTPs mixed + dUTP + $MgCl_2$ was supplied by Roche. The SyberGreen (SG) IL28B probes and primers designated by Drs. Clewley and Foster were authorised, and the RFH was supplied by Applied Biosystems. The Tris/EDTA (TE) buffer for the reconstitution and dilution were used from Sigma.

c- Controls

Positive control samples (5 µl of 1:100 dilution) were used in duplicate with each run.

d- Primer and probes were supplied by *Invitrogen* in 100 µM

SNP	Primer & probes	Allele	Sequence (5' → 3')
rs12979860C/T	Spain-Fwd		GCCTGTCGTGTACTGAACCA (F60)
rs12979860C/T	Spain-Rev		GCGCGGAGTGCAATTCAAC(R60)
rs12979860C/T	Spain-P1	C	VIC-TGGTTCGCGCCTTC
rs12979860C/T	SpainP2	T	FAM-CTGGTTCACGCCTTC
rs809917G/T	917-Fwd		TGTCACTGTTCCTCCTTTTGTTTT(F17)
rs809917G/T	917-Rev		TACAGCATGGTTCCAATTTGG (R17)
rs809917G/T	917-P1	G	VIC-TGTGAGCAATGTC
rs809917G/T	917-P2	T	FAM-CTGTGAGCAATTTC

d- Reaction

Primers and probes received in the form of frozen dry pellets were then reconstituted to 100 µM using 1X TE buffer and were aliquoted in 10 µM and then stored at -20°C for working strength. Reactions were then prepared for the two SNP runs, separated by preparing the master mix first according to the outlined procedure.

For rs12979860C/T, the PCR reaction was prepared for 100 reactions by using 1263µl of the MM in 76 µl of both F60+R60 and 13 µl from both P1+P2 and 150 µl of SG all in 431 µl of molecular biology grade (MG) H_2O. Then, the mixture was aliquoted in 20 µl in the plate with 5 µl of the extracted DNA and controls in each well.

For rs809917G/T, the PCR reaction was prepared for 100 reactions by using 1263 µl of the MM in 76 µl of both F17+R17 and 13 µl of P1 and 5.5 µl of P2 and 150 µl of SG all in 329 µl of molecular biology grade (MG) H_2O. The mixture was then aliquoted in 20 µl into the plate with 5 µl of the extracted DNA and the controls in each well.

Results
KSA HCV Results

The mean age for the 114 patients was 45 ± 13 years. The study produced results from 65 males and 49 females. Most of the genotypes (87%) were genotype 4. The mean viral load was 498158 ± 109318.4. The frequency of SNPs of IL28B for genotype RS-60 in the HCV patients was 22.8% for CC, 54.4% for CT and 22.8% for TT. The frequency for genotype RS-17 was 6.1%, 66.7% and 27.2% for GG, GT and TT, respectively. The data showed that 29 (25.43%) of the 114 patients with HCV achieved SVR and became responders (R). Amongst the responding patients, 22.6% of them were genotype CT, while 19% were CC and 38% were TT. There was a significant correlation between the IL28B genotype frequency of RS-60 and the treatment response ($p < 0.001$). The remaining 52 (46%) patients who failed to respond (NR) had genotype frequencies of 53% CT, 50% CC and 23.1% TT. The correlation between NR and IL28B genotype frequencies in RS-60 was statistically significant ($p < 0.001$). The present study also included partial responders (PR) (N=6) and transitional

responders (TR) (N=13). The PRs had similar frequencies of CC and TT genotypes (7.7%), which were higher than the frequency of CT genotype (3.2%). Amongst the PRs, the correlation between IL28B genotypes frequency and RS-60 was statistically significant (p = 0.026). Among the TRs, the CT genotype frequency was identified in approximately 8%, which was lower than the frequencies of the CC genotype (23.1%) and the TT genotype (23.1%). The overall frequencies of the IL28B genotypes for RS-60 did not show any significant correlation with the TR group (p =0.103). The data did not support a correlation between treatment response and the IL28B genotypes frequency for RS-17. Approximately 46% of the NRs were IL28B genotype GT for RS-17, 48.4% were TT and 28.6% were GG. Amongst the SVR patients, there were similar frequencies of GG and GT (28.6% and 28.9%, respectively), which were higher than that of TT (approximately 16%).

KSA HBV Results

These data included 101 patients infected with HBV. The general characteristics of the HBV patients are summarised in Table 4. The mean age was 39.40 ± 12.48 years. Of the 101 patients, there were 55 males (54.45%). The viral load was 5.1 ± 2.7 (log IU/mL)**.** The frequencies of IL28B genotypes for RS-17 were 94.1% with GT, 2.9% with, TT and 2% with GG. However, the IL28B genotype frequencies for RS-60 were 67.6% with CT, 21.6% with CC and 9.8% with TT. Amongst the 101 patients, 47 (46.53%) achieved SVR. Amongst the IL28B genotypes for RS-60, the CT genotype was the most common (50.1%), followed by the CC genotype (40.9%) and the TT genotype (30%). The relationship between R and IL28B genotype frequency for RS-60 was not statistically significant (p= 0.312). Among the NRs (N=26), the CC genotype frequency was the highest (36.4%), followed by CT (23.2%) and TT (20%). There was no significant correlation between the IL28B genotype frequency for RS-60 and NR (P = 0.125). Amongst the PRs (N=25), the TT genotype was the most common (50%), followed by similar frequencies of CT and CC (21.7% and 22.7%, respectively). No significant correlation was found between PR and the IL28B genotype frequency (RS-60) (P= 0.103). The output data no significant correlation between the IL28B genotype frequencies for RS-17 and treatment response variables (P >0.05 for all variables). In the NR group, the TT genotype was most common (66.3%), followed by GT (25%), and GG was not identified in any of the cases. In the R group, GG was identified in all of the cases (100%), followed by GT (46.9%), with no cases identified with the TT genotype . The PR group showed the highest frequency of TT (33.3%), followed by GT (25%); no cases were identified with the GG genotype.

KSA HIV Results

This study also included 100 patients with HIV. The mean age was 39.30 ± 12.18 years. Males outnumbered females (86% and 14%, respectively). The mean viral load was 1.45 ± 4.3 (log IU/mL). The frequencies of IL28B genotypes for RS-17 were 19% with GG, 54% with GT and 27% with TT, whereas for the IL28B genotypes for RS-60, the CT genotype was the most common (73%), followed by the CC (17%) and TT (10%) genotypes. By treatment response, 22% of the patients were responders, 47% were non-responders, 22% were partial responders and 10% had responses that could not be determined. Treatment response and the frequency of IL28B for RS60 genotypes were significantly correlated. Twenty-one per cent of the patients achieved SVR, and amongst them, the CT genotype was the most common (26%), followed by the CC genotype (11.8%). The relationship between the IL28B for RS60 genotypes and the treatment response was statistically significant ($p = 0.001$). Forty-seven per cent of the HIV patients did not respond to treatment. Amongst them, the CC genotype was the most common (77%), followed by the TT (50%) and CT (40%) genotypes. The relationship between IL28B for RS60 genotypes and NR was statistically significant ($P = 0.023$). Amongst the PRs, the TT genotype was the most common (40%), followed by the CT (23.3%) and CC (5.9%) genotypes. The relationship between IL28B for RS60 genotypes and PR was statistically significant ($P = 0.002$). The frequency of RS-17 genotypes was significantly associated with the patients who did not achieve response ($P = 0.010$), with the GT genotype being the most common (57%), followed by the GG (53%) and TT (22%) genotypes. The HIV patients with the RS-17 genotype did not show any significant variation ($P = 0.052$ for both).

UK-HCV Results

The study included 13 patients with HCV from the UK (one patient's samples were missing). The mean age of the patients was 64.23 ± 8.94 years. Most of the patients (69%) were female. The viral load was 2.98 ± 7.0 log IU/mL. The levels of AST and ALT were 45.23 ± 4.1 and 49 ± 4.33 U/L, respectively. The frequency of IL28B genotypes RS-17 showed the predominance of the GT genotype (69%), followed by the TT (30.7%) and GG (7.69%) genotypes. The frequency of the IL28B genotypes for RS-60 showed the predominance of the CT genotype (62%), followed by the CC (31%) and TT (8%) genotypes. Over half of the patients (54%) did not achieve SVR. Twenty-four per cent of the patients achieved SVR, while another 24% were transitional responders (TR). Amongst them, three participants who achieved SVR had TT (100%), CC (25%) and CT (12.5%) genotypes, while 7 non-responding participants had CT (37.5%) and CC (50%). None of the non-responding

cases were identified with the TT genotype. No significant correlation was observed between the RS-60 genotype and treatment response ($p > 0.05$ for all variables), likely due to the small number of patients from the UK. The SVR patients had the GT (11.11%), TT (33.3%) and GG (100%) genotypes. The GT and TT genotypes (33.3%) were equally common amongst participants who failed to achieve SVR. All of the transitionally responding patients (TR) had the GT genotype. However, no significant correlations were observed between the treatment response and the frequency of the RS-17 genotype ($P > 0.05$).

Relationship between viral load and frequency of IL28B genotypes

Tables 1 explored both the frequency of the IL28B genotypes investigated in this study and whether they were associated with the HCV viral load. There were no significant correlations observed from either of the IL28B genotypes ($P = 0.388$) amongst the UK patients. However, the importance of this finding is limited due to the small sample size. The RS-17 genotype in the KSA-HIV/HCV co-infected patients was significantly associated with HCV viral load ($P=0.034$). The GT genotype (61.2%) was associated with a higher viral load, followed by TT (23.9%) and GG (14.9%), while the GT and TT genotypes were more common (32.1% and 39.3%, respectively) and associated with lower viral loads. The frequency of GT was lower (32.1%) in patients with a low HCV viral load compared with the frequency in those with a higher viral load (61.2%). In the Saudi patients with HIV, the RS-17 GT genotype was associated with a higher viral load, whereas patients with the GG genotypes had lower HCV loads. There was no association between the RS-60 genotype of the HIV-HCV co-infected patients with HCV load ($P=0.230$). In addition, there were also no positive correlations between the RS-17 and RS-60 genotypes in the HBV-HCV co-infected patients. Finally, no correlations were found between the RS-17 and RS-60 genotypes in the HCV-infected patients and the HCV viral load ($P = 0.904$ and 0.712, respectively).

Table 1: Statistical relationships between HCV viral load and genotype frequencies in HCV-, HBV- and HIV- infected patients from KSA and HCV-infected patients from the UK.

Variable	RS-17					RS-60				
	Viral load	GG (N,%)	GT(N,%)	TT (N,%)	P value	Viral load	CC(N,%)	CT(N,%)	TT (N,%)	P value
KSA-HCV	≤1000	3 (6.7%)	29(64.4%)	13 (28.9%)	0.904	≤100000	8(17.8%)	27 (60%)	10(22.2%)	0.71 2
	>1000	3 (8.3%)	24(66.7%)	9 (25%)		>100000	9(25%)	19(52.8%)	8 (22.2%)	
KSA-HBV	≤1000	1 (1.9%)	51(69.2%)	1 (1.9%)	0.483	≤1000	13(24.5%)	34(64.2%)	6(11.3%)	0.880
	>1000	1 (3.3%)	27 (90%)	2 (6.7%)		>1000	6 (20.9%)	20(66.7%)	4(13.3%)	
KSA-HIV	≤1475	8 (28.6%)	9 (32.1%)	11(39.3%)	0.034	≤1475	2(7.1%)	22(78.6%)	4(14.3%)	0.230
	>1475	10(14.9%)	41(61.2%)	16(23.9%)		>1475	14(20.9%)	47(70.1%)	6 (9%)	
UK-HCV	≤380269	0 (0%)	3 (75%)	1 (25%)	0.388	≤380269	1 (25%)	3 (75%)	0 (0%)	0.388
	>380269	1(33.3%)	1(33.3%)	1 (33.3%)		>380269	1 (33.3%)	1 (33.3%)	1(33.3%)	

Discussion

This study addressed the SNPs in the IL28B gene that were previously associated with poor response to HCV therapy with IFN-α and IFN-α + RBV in a cohort of Saudi Arabian patients with either HCV mono-infection or co-infection with HBV or HIV and compared them with the genotypes of a small group of UK patients previously analysed for mutations in the ISDR region. The distribution of the IL28B polymorphisms was comparable between the two groups, although the RS-17 GT genotype was more common amongst the HCV-HBV co-infected patients (Figure 1, P=0.05). The RS-17 GG and TT genotypes were present at lower frequencies (P=0.03 and P=0.02, respectively) (Figure 1). A comparison of our results with those of other studies is shown in (Figure 2). This study also showed that the predominant HCV genotype in the Kingdom of Saudi Arabia was genotype 4 (87%), which is consistent with WHO data.

With regard to treatment response, 25.43% of the 114 patients with HCV mono-infection achieved SVR. (Figures 3) (Figure 4) summarise the response to therapy and IL28B genotypes. The RS-60 CC genotype was present in only 19% of the SVR group, whereas the CT genotype was identified in 22.6% and TT in 38.4%. The patterns of the rs12979860 genotype frequencies in the present study are not consistent with those of a majority of other studies. Hendy et al. (2011) reported a higher frequency for the CC (87.2%) genotype of RS-60 amongst patients who achieved SVR, which was significantly higher compared with the

CT (25.5%) and TT (10%) genotypes. This finding was supported by additional previous studies (Suppiah et al., 2009; Tanaka et al., 2009; Ge et al., 2009).

The significant correlation between RS-60 TT genotype and response to therapy (P<0.001) supported prior results obtained by Aparicio et al. (2010). The partially responding (PR) and transitionally responding (TR) groups had the CC genotype at a higher frequency (15.4%) than the CT genotype. The TT genotype frequency was amongst the lowest (7.7%). However, the frequency in the NR was 32.3% for the TT genotype and 42.9% for the GG genotype (Figures 5, 6 and 7).

In the HCV-HBV co-infected group, the frequency of RS-17 GT was dominant at 94.1% and was significantly more common than the TT (2.9%) and GG (2%) genotypes, while the frequency of RS-60 CT was 67.6%, CC was 21.6% and TT was 9.8%. The response to HCV treatment in this group (46.53%) was higher than in the mono-infected group, although there was no strong correlation with response to therapy. Therefore, the data did not support a role for RS-17 (p > 0.05) or RS-60 (P > 0.05) (Figure 8). Other studies have shown that the CC genotype at RS-60 was not associated with HBV recovery or that the SNPs were not associated with the patients who were either likely to have a response or those with the NR phenotype (Martin et al., 2010). The authors concluded that the IL28B SNP affects the immune response to HCV but not to HBV.

However, the lack of association between IL28B and response to HBV is unexpected because IL28B stimulates ISGs, which play an important role in the immune response to HIV and HBV infections. Furthermore, in HBV transgenic mice, ISGs are considered a main mechanism of non-cytolytic inhibition of HBV replication (Guidotti, 2002). Additionally, Robek (2005) reported that exogenous murine interferon-λ2 (IL28A) added to an immortalised murine hepatocyte cell line that normally allows HBV infection and replication resulted in an inhibition of HBV replication by >90%, and this inhibition was thought to be mediated by an up-regulation of ISGs. However, we did not evaluate the HBV antiviral responses in this study.

The HCV-HIV co-infected group had RS-60 genotype frequencies that were associated with HCV treatment response (P = 0.01), while the RS-17 genotype frequencies were not associated with response (P >0.05). Thus, the data suggest the possibility of using the RS-60 SNP to predict HCV treatment response in these Saudi patients, although further studies are necessary to confirm this association. On the other hand, the RS-17 genotype in the HIV-infected patients from KSA was significantly correlated with the HCV viral load (P = 0.034). More HCV-HBV co-infected patients achieved SVR (46.5%) compared with HCV

mono-infected patients (25%), and this was lower than observed in HIV co-infection (21%) (Figure 9). This study presented a suitable opportunity to test samples from HCV-infected patients from the UK that were already assessed for mutations in the ISDR, as described previously. Examination of the RS-17 showed a predominance of the GT genotype (69%), followed by the TT (30.7%) and GG (7.69%) genotypes. By contrast, for the IL28B genotypes RS-60, the CT genotype was predominant (62%), followed by the CC (31%) and TT (8%) genotypes. In conclusion, this study provided new data on the distribution of the IL28B genotypes in the Saudi Arabian HCV-infected population and may be useful in identifying the IL28B genotypes that identify the patients who will have better responses to anti-HCV therapy.

Distribution of IL28B polymorphisms (rs60 and rs17) in HCV co-infected patients
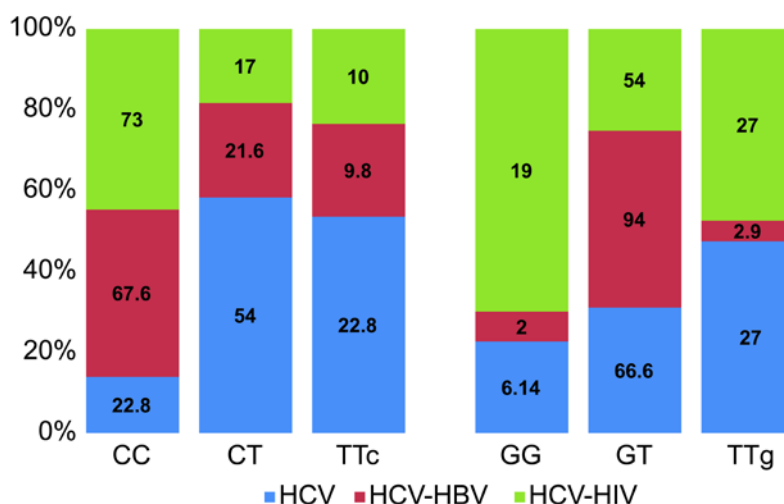
Figure 1 The distribution and frequencies of IL28B polymorphisms for rs60 and rs17 in HCV mono-infected HCV and co-infected patients

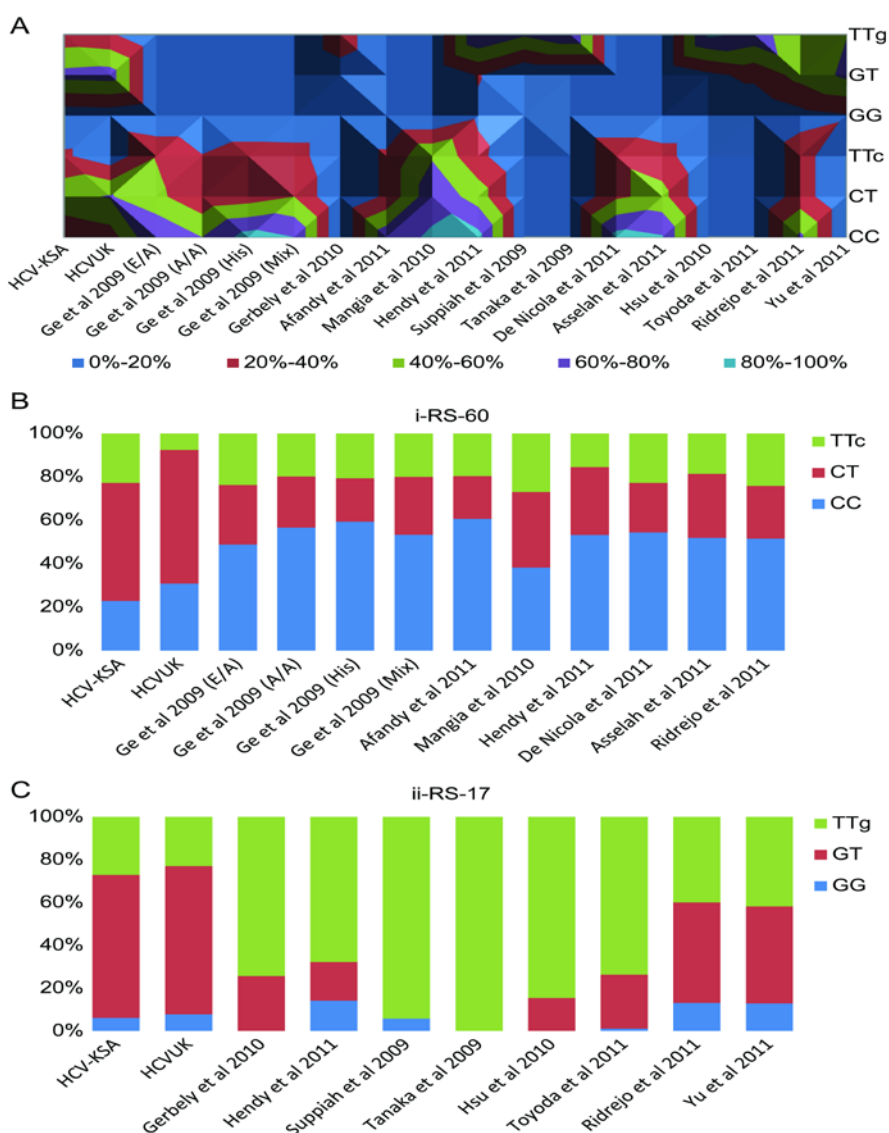**Comparison of polymorphisms between KSA and UK**
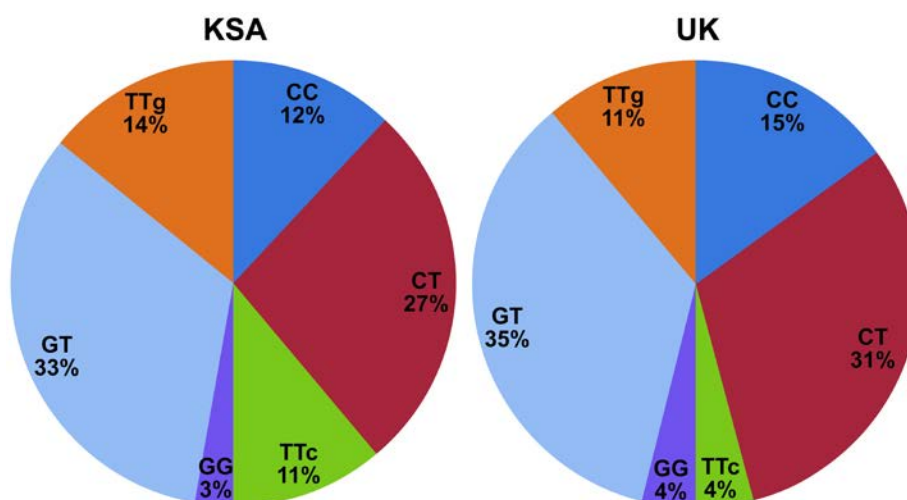
Figure 2 A comparison of the RS-17 and RS60 polymorphisms between the KSA and UK.

**Comparison of the response to treatment between the KSA and UK**
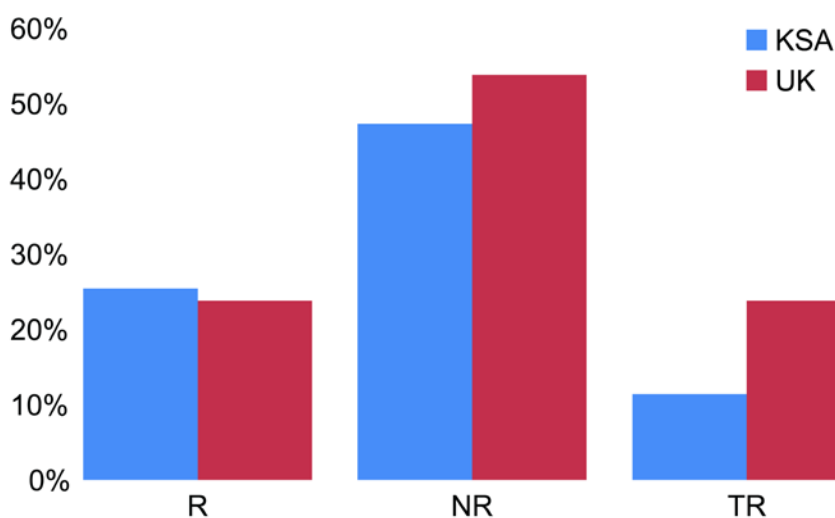
Figure 3   A comparison of the responsiveness to treatment between the two countries, showing the convergence at R and the variance of NR and TR.

RS-17 and RS-60 surface comparison

Figure 4 A statistical surface comparison (panel A) and the prevalence of genotypes of RS-60 (panel B) and RS-17 (panel C) between our study and other previous published studies, showing that Ge et al. in 2009 found that CC in the responding patients applied to 80% of European-Americans, 60% of African-Americans and 89% of Hispanics, amongst whom 45%, 25% and 50%, respectively, had the CT genotype and 39%, 21% and 31%, respectively, had the TT genotype. By contrast, findings from others suggested between 64% and 87% have the CC genotype, between 30% and 75% have the CT genotype and between 10% and 37% have the TT genotype.
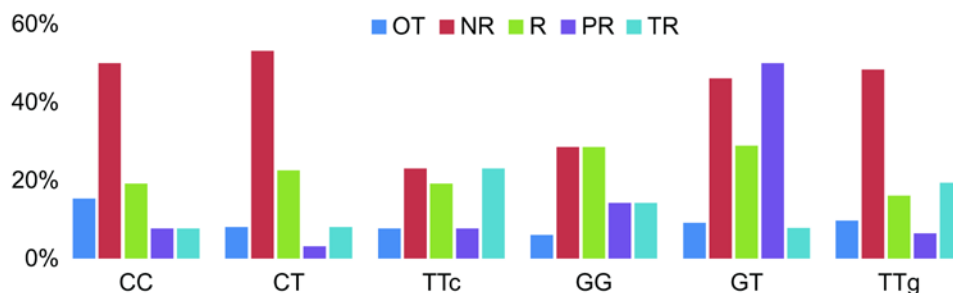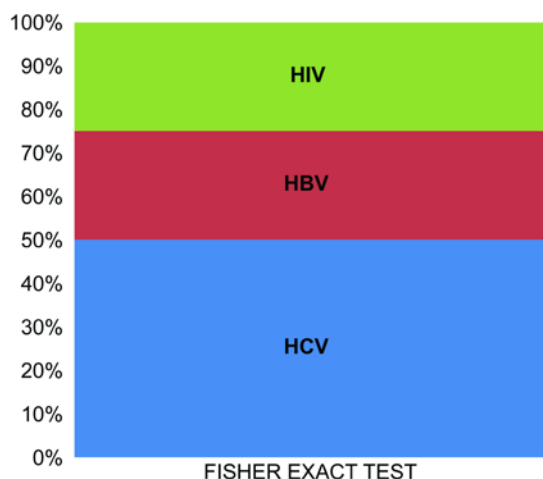

Figure 5 A comparison of IL28B polymorphisms in KSA HCV patients.
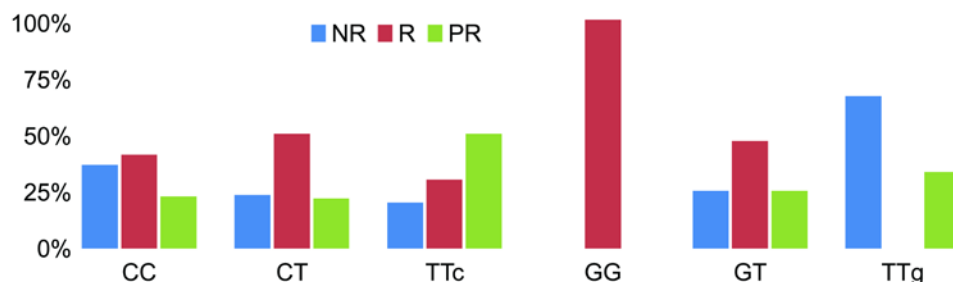
Figure 6 A comparison of IL28B polymorphisms in KSA HBV patients by their response to therapy.
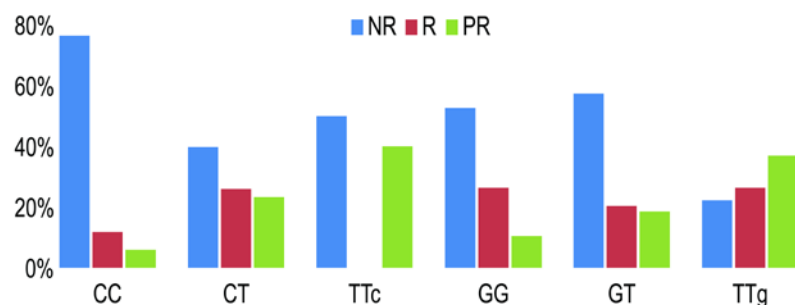
Figure 7 A comparison of IL28B polymorphisms in KSA HIV patients by their response to therapy.

Figure 8 The Fisher's exact test shows a negative significant relationship between RS60-RS17 in HCV, HBV and HIV KSA patients.

Conclusion

In summary, this study provided new data on the distribution of IL28B genotypes in Saudi Arabian HCV-infected patients and demonstrated the usefulness of identifying IL28B genotypes to identify patients who may or may not respond to anti-HCV therapy.

A comparison of polymorphisms amongst mono-infected KSA patients
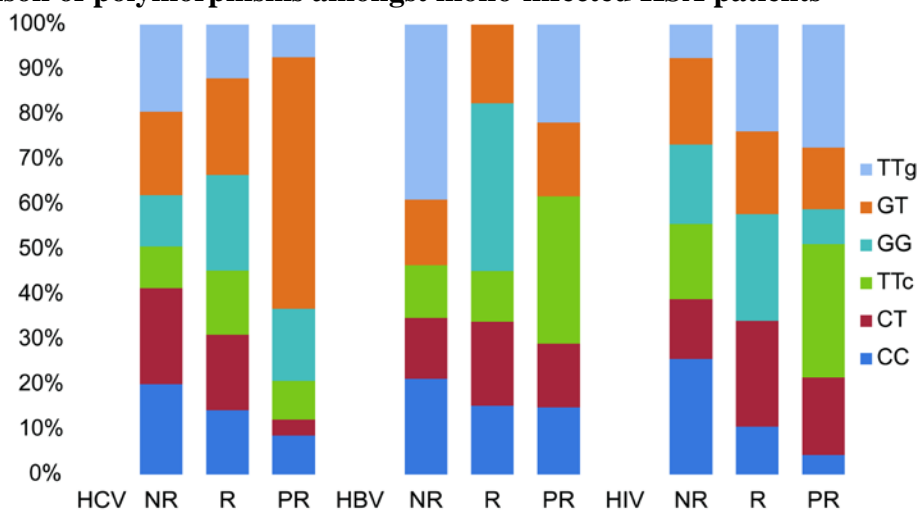
Figure 9 A comparison of responsiveness in mono-infected patients, showing the frequency changes in the RS60 and RS17 polymorphisms.

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