ORIGINAL ARTICLE

FokI VITAMIN D RECEPTOR POLYMORPHISM AS A PREDICTOR OF RESPONSE TO HEPATITIS C ANTIVIRAL TREATMENT

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Background: FokI vitamin D receptor (VDR) genotype ff has been debated as predictor of response to treatment. This study was designed to find out the association of FokI VDR polymorphism with response to chronic hepatitis C daclatasvir- and sofosbuvir-based treatment. **Methods:** This case control study was conducted at Federal Postgraduate Medical Institute, Lahore from Jan 2019 to Apr 2021. It included 66 chronic hepatitis C genotype 3 patients who responded to daclatasvir and sofosbuvir-based treatment (with ribavirin for cirrhotic patients) and attained sustained virologic response (SVR) three months after completion of treatment, and 66 gender and age matched chronic hepatitis C genotype 3 patients who did not respond to the treatment. Demographic data was collected and 3 mL of blood was drawn from each participant. DNA extraction was done followed by PCR-restriction fragment length polymorphism. Samples were run on 12% polyacrylamide gel and visualized under UV light. Data was analysed using SPSS-24. **Results:** Frequencies of FokI VDR genotypes FF, Ff, and ff were 54.5%, 28.8%, and 16.7% in responders, and 60.6%, 36.4%, 3.0% in non-responders. There was a significant association between FokI VDR polymorphism and response to treatment (p=0.03). No significant association was found between FokI polymorphism and cirrhosis. Logistic regression showed FokI genotype ff to be a significant predictive factor for a SVR (p=0.041). **Conclusion:** FokI VDR polymorphism is associated with response to daclatasvir- and sofosbuvir-based antiviral treatment in chronic hepatitis C genotype 3 patients. FokI genotype ff could be considered as a predictive marker for response to treatment.

Keywords: Vitamin D receptor, VDR Polymorphism, Hepatitis C, Polymorphism, Cirrhosis, Anti-viral

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INTRODUCTION

Hepatitis C is a worldwide health problem. It is estimated that 58 million individuals suffer from chronic hepatitis C infection globally and there are 1.5 million new cases every year.[1] About 20% of chronic hepatitis C patients progress to end stage cirrhotic liver disease or hepatocellular carcinoma.[2] Pakistan has a high prevalence of 5% nationwide[3], and despite the advent of the directly acting antiviral treatment, hepatitis C prevalence is still persistent.[4]

Vitamin D receptor is a nuclear receptor that acts as a transcription factor. The receptor binds to the active vitamin D and mediates its actions.[5,6] It is encoded by the vitamin D receptor gene on chromosome 12q.[7] Recent studies have shown association of genetic variations in the VDR gene with susceptibility as well as chronicity of hepatitis C infection.[8] These genetic variations have also been studied in relation to the response to pegylated interferon and ribavirin treatment.[9,10]

El-derany *et al*[11] found a significant association between FokI VDR polymorphism and the response to pegylated interferon with ribavirin in CHC genotype 4 patients, whereas Wang *et al*[12] found no significant association between FokI genotypes and the response to treatment. García-Martín *et al* reported FokI VDR genotype ff as a predictor of the response to treatment.[13]

There is a need to study the association of FokI VDR polymorphism with the new directly acting antiviral treatment of hepatitis C virus as pharmacogenetics are different for the different drugs. Considering that, the controversial results of the previous studies, and the proposed importance of FokI VDR polymorphism in predicting the outcome of treatment, this study was designed to find out the association of FokI VDR polymorphism with the response to daclatasvir and sofosbuvir (with ribavirin for cirrhotic patients).

METHODOLOGY

This was a case control study conducted in the department of physiology, Federal Postgraduate Medical Institute from January 2019 to April 2021 after approval from the Institutional Ethical Review Board. Sample size was calculated to be 25 responders to hepatitis C antiviral treatment and 25 non-responders to hepatitis C antiviral treatment at 95% confidence interval and 5% margin of error with expected FokI vitamin D receptor polymorphism frequency of 40% and 90% in responders and non-responders respectively.[14] However, 132 subjects were enrolled (66 responders to hepatitis C antiviral treatment and 66 non-responders to hepatitis C

antiviral treatment) by non-probability convenient sampling from Shaikh Zayed hospital, Lahore. Subjects included males and females aged ≥18 years with cirrhotic/non-cirrhotic HCV liver disease who received directly acting antiviral drugs (daclatasvir and sofosbuvir with or without ribavirin), and did the HCV-RNA test 12 weeks after completion of treatment.

Responders were those who maintained sustained virologic response 12 weeks after completion of treatment (Hepatitis C virus-RNA negative). Non-responders were those who did not maintain sustained virologic response 12 weeks after completion of treatment (Hepatitis C virus-RNA positive). Both groups were age and gender matched.

Patients with autoimmune hepatitis, alcoholic liver disease, hepatitis B surface antigen, HIV, hepatocellular carcinoma, decompensated liver cirrhosis, severe renal disorder, uncontrolled diabetes, uncontrolled hypertension, or severe depression were excluded. A written informed consent was taken from each participant and demographic data was recorded. Clinical reports of platelet count, haemoglobin level, prothrombin time (PT), International Normalized Ratio (INR), and liver function tests (LFTs) were recorded.

About 3 mL of blood was drawn from each patient through venipuncture. At School of Biological Sciences (University of the Punjab), DNA extraction from blood was done followed by amplification of the DNA fragment of vitamin D receptor gene containing the FokI restriction site (rs2228570), and then restriction fragment length polymorphism (RFLP) analysis.

DNA extraction was done using the extraction kit (ThermoScientific #K0781) and stored at -20 °C. The DNA fragment containing the FokI VDR polymorphism (rs2228570) was amplified using the forward primer 5′-AGCTGGCCCTGGCACTGACTC TGGCTCT-3′ and the reverse primer 5′-ATGGAAAC ACCTTGCTTCTTCTCCCTC-3′[15] in a 20 µL PCR mixture as follows:

3 µL 2.5 mM dNTPs, 2 µL 10× NH_4SO_4 buffer, 3 µL 25 mM $MgCl_2$, 1.5 µL 10 µM forward primer, 1.5 µL 10 µM reverse primer, 0.5 µL 5 U/µL Taq polymerase, 5 µL DNA, and 3.5 µL water.

The PCR reaction for amplification of the PCR product containing FokI SNP (rs 2228570) underwent 35 cycles consisting of initial denaturation for 5 minutes at 95 °C, denaturation for 30 seconds at 95 °C, annealing for 45 seconds at 68 °C, extension for 45 seconds at 72 °C, and then final extension for 10 minutes at 72 °C. Genotyping was done by restriction fragment length polymorphism using restriction enzyme FokI (FastDigest Thermo Scientific #FD2144), i.e., 10 µL of PCR (0.2 µg), 2 µL 10× FastDigest Green Buffer, 1 µL FastDigest enzyme, and 17 µL nuclease free water were added and mixed. The 30 µL mixture was incubated at 37 °C in a heat block for 5 minutes followed by inactivation for 5 minutes at 65 °C and then samples were run on 12% polyacrylamide gel and visualized under UV light.

Data was entered and analysed using IBM SPSS-24. Comparison of age, BMI, platelets count, haemoglobin, total bilirubin, direct bilirubin, Aspartate aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline phosphatase (ALP), serum albumin, PT, and INR in responders and non-responders was done using *t*-test (for normally distributed data) and Mann-Whitney test (for not-normally distributed data). Frequencies of the FokI VDR polymorphism genotypes were studied in accordance with the Hardy-Weinberg equilibrium. Association of FokI VDR polymorphism with the response to treatment was studied using chi-square test. Association of FokI VDR polymorphism with cirrhosis was studied using chi-square test in responders group and Fisher's exact test in non-responder group respectively. Binary regression was used to assess the association between the different independent variables and the response to treatment, and $p<0.05$ was considered statistically significant.

RESULTS

There were 40 males and 26 females in each group with Mean age of 50.03±7.53 years in the responders group and 49.02±7.54 years in the non-responders group. No significant differences in gender and age were found between the two groups (p=1 and 0.36 respectively). No significant differences were found in BMI, platelet count, haemoglobin, PT, INR, serum albumin, total bilirubin, direct bilirubin, AST, ALT, and ALP in responders *vs*. non-responders ($p<0.05$) (Table-1).

The RFLP analysis showed a single band of 267 base pairs in the wild homozygous FF genotypes (CC), two bands of 208 and 59 base pairs in the mutant homozygous ff genotypes (TT), and three bands of 267, 208, and 59 base pairs were seen in the heterozygous Ff genotypes (CT). The frequency of FokI VDR genotypes, FF, Ff, and ff was 36 (54.5%), 19 (28.8%) and 11 (16.7%) in responders and 40 (60.6%), 24 (36.4%) and 2 (3.0%) in non-responders. Chi-Square test showed a significant association between FokI VDR polymorphism genotypes and response to treatment (Chi-Square=7.023, p=0.03). There was no significant association between FokI VDR polymorphism and cirrhosis in responders and non-responders ($p<0.05$) (Table-2). Moreover, there was no significant association between FokI VDR polymorphism and gender (Table-3).

Logistic regression showed FokI genotype ff as a significant predictive factor for response to treatment (p=0.41, OR=0.179, 95% CI=0.034–0.929) (Table-4).

Table-1: BMI, platelet count, haemoglobin, prothrombin time (PT), INR, and liver function tests of responders and non-responders

Variables	Responders Mean±SD	Non-Responders Mean±SD	*p*
BMI (Kg/m^2)	26.97±6.81	28.01± 6.46	0.369[a]
Platelets (×10^3/µL)	246.98±70.69	234.55±73.78	0.294[b]
Haemoglobin (g/dL)	13.15±1.97	12.74±1.96	0.223[b]
Prothrombin time (Sec)	14.89±0.86	14.68±0.81	0.093[b]
INR	1.14±0.19	1.14±0.16	0.939[b]
Serum albumin (g/dL)	3.52±0.58	3.45±0.55	0.578[b]
Total bilirubin (mg/dL)	0.97±0.57	1.00±0.56	0.571[b]
Direct Bilirubin (mg/dL)	0.43±0.38	0.44±0.38	0.707[b]
AST (U/L)	64.41±17.29	65.86±16.78	0.554[b]
ALT (U/L)	69.42±22.06	70.95±22.21	0.692[a]
ALP (IU/L)	108.17±21.53	109.82±20.48	0.652[a]

[a]Student's *t*-test for normally distributed data
[b]Mann-Whitney test for not normally distributed data

Table-2: Association of FokI VDR polymorphism with cirrhosis

	Responders		Non-Responders	
FokI Genotype	Cirrhotic (n=33)	Non-cirrhotic (n=33)	Cirrhotic (n=33)	Non-cirrhotic (n=33)
FF	17	19	21	19
ff	5	6	0	2
Ff	11	8	12	12
Chi-Square/Fisher's exact test	0.676[a]		1.751[b]	
p	0.713		0.592	

[a]Chi-square test, [b]Fisher's exact test

Table-3: FokI VDR polymorphism genotypes in males and females [n (%)]

VDR Polymorphism Genotype		Responders		Non-Responders	
		Male	Female	Male	Female
FokI	FF	24 (60)	12 (46.2)	25 (62.5)	15 (57.7)
	ff	4 (10)	7 (26.9)	2 (5.0)	0 (0.0)
	Ff	12 (30)	7 (26.9)	13 (32.5)	11 (42.3)
	Total	40 (100)	26 (100)	40 (100)	26 (100)
	Chi-Square/Fisher's exact test	3.313[a]		1.422[b]	
	p	0.191		0.512	

[a]Chi-square test was used, [b]Fisher's exact test was used

Table-4: Logistic regression analysing potential predictors of SVR in chronic hepatitis C patients

Variables	B	*p*	OR (95% CI)
FF	Referent		
ff	-1.721	0.041*	0.179 (0.034–0.929)
Ff	-0.033	0.939	0.968 (0.418–2.239)
Age	-0.022	0.406	0.979 (0.930–1.030)
Gender	0.219	0.606	1.245 (0.542–2.857)
Smoking	-0.516	0.320	0.597 (0.216–1.652)
BMI	0.019	0.542	1.019 (0.959–1.082)
Platelets	-0.003	0.339	0.997 (0.992–1.003)
Haemoglobin	-0.143	0.163	0.867 (0.709–1.060)
Cirrhosis	-0.106	0.860	0.900 (0.278–2.908)
Total bilirubin	0.241	0.798	1.273 (0.201–8.055)
Direct bilirubin	-0.291	0.853	0.747 (0.034–16.203)
AST	-0.002	0.907	0.998 (0.970–1.027)
ALT	0.006	0.534	1.006 (0.986–1.027)
ALP	0.002	0.867	1.002 (0.982–1.022)
Serum albumin	-0.191	0.646	0.826 (0.366–1.864)
Prothrombin time	-0.301	0.292	0.740 (0.423–1.295)
INR	0.028	0.986	1.029 (0.041–25.749)

*Significant

DISCUSSION

Our study found a significant association between FokI VDR polymorphism and the response to DAAs (Daclatasvir and sofosbuvir with/without ribavirin) in HCV genotype 3 patients with a protective role (in favour of response to treatment) of ff genotype. These results were in line with the results of El-Derany *et al*[11] whose study showed a significant association of FokI VDR polymorphism genotypes with the response to pegylated interferon with ribavirin and showed the homozygous FokI ff genotype to predict SVR in hepatitis C patients. In their regression analysis, the mutant 'f' allele was significantly associated with responding to the treatment and achieving SVR.[11]

On the contrary, Arai *et al*[16] found no significant association between FokI VDR polymorphism and the response to hepatitis C treatment. Their study was on genotype 1 chronic hepatitis C patients and the hepatitis C genotype could itself influence the response to treatment, thereby influencing the impact that a VDR polymorphism might have.

Though, Cusato *et al* and Abdelsalam *et al* found an association between FokI polymorphism and response to pegylated interferon and ribavirin, ff genotype was associated with relapse rather than response to treatment.[17,18] Similar to our results, García-Martín *et al* also reported the mutant 'f' allele of FokI VDR polymorphism to be significantly associated with achieving SVR and related inversely with failure of therapy in chronic HCV patients.[13]

The mechanism by which FokI VDR polymorphism influence the response to treatment is not known. It has been suggested that the two alleles 'F' and 'f' result in the production of two VDR variant proteins which function as transcription factors and their interaction with other co-transcription factors might cause an organ-specific/cell-specific expression that is different for the different VDR variant proteins.[19] FokI VDR polymorphism resulted in different effects on immunity and that was seen at the transcriptional activity level as well as at the level of cytokine synthesis and proliferation by immunity cells.[20]

The above mentioned studies were conducted on patients who had received pegylated interferon and ribavirin, whereas our study is reporting on the association of FokI VDR polymorphism with the response to DAAs (Daclatasvir and sofosbuvir with or without ribavirin) in HCV genotype 3. FokI VDR polymorphism could well be considered as a new marker for the prediction of response to treatment in chronic hepatitis C patients.

CONCLUSION

There is a significant association between FokI VDR polymorphism and the response to the daclatasvir and sofosbuvir-based antiviral treatment in chronic hepatitis C genotype 3 patients. FokI homozygous mutant ff genotype could be used to predict the response to treatment.

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