ApaI VDR Polymorphism as a Risk Factor of Treatment Failure in Chronic Hepatitis C Patients

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ABSTRACT

The objective of this study was to find out the association between ApaI vitamin D receptor (VDR) polymorphism and the response to hepatitis C directly acting antiviral treatment. This study which is a case control study included 66 hepatitis C patients (genotype 3) who responded to the directly acting antiviral treatment and achieved negative HCV-RNA three months after completing the treatment (sustained virologic response (SVR)) and 66 hepatitis C patients (genotype 3) who did not achieve SVR three months after completing the same treatment. Informed consent was taken from participants. Demographic data was collected, and 5 mL of blood was drawn from each participant and used for DNA extraction, polymerase chain reaction and restriction fragment length polymorphism analysis. After restriction, samples were run on 2% agarose gel followed by visualization under UV light. Data analysis was done using IBM SPSS 24. We found that the distribution of ApaI genotypes was 28 (42.4%), 27 (40.9%), and 11 (16.7%) for the genotypes AA, Aa, and aa in responders and 22 (33.3%), 26 (39.4%), and 18 (27.3%) in non-responders. The allelic distribution was 83 (62.9%) and 49 (37.1%) for the “A” and “a” alleles in responders and 70 (53%) and 62 (47%) in non-responders. ApaI genotype “aa” was found to be a significant predictor of treatment failure (p-value= .024, OR= 3.589, 95% CI= 1.181-10.911). There was no significant association between ApaI VDR genotypes and cirrhosis and ApaI VDR genotypes and gender (p-values < .05). To conclude ApaI genotype aa could be used as a marker to predict treatment failure in hepatitis C patients receiving directly acting antiviral treatment.

INTRODUCTION

Hepatitis C virus is an important worldwide health problem (Stanaway et al., 2016). It is a main cause of cirrhosis, hepatocellular carcinoma, and mortality (Perz et al., 2006). Hepatitis C virus is also shown to cause complications beyond the liver such as lymphoma, diabetes, and chronic renal disease (Younossi et al., 2016). It is estimated that approximately 71 million individuals are infected with hepatitis C virus worldwide (Polaris Observatory HCV Collaborators, 2017). More than 50% of HCV infections are in China, Pakistan, Egypt, Nigeria, Russia, and India (Gower et al., 2014).

In Pakistan, the prevalence of hepatitis C virus is the 2nd highest in the world (Hill et al., 2017; Abbas and Abbas, 2020) and is about 5% nationwide (Al Kanaani et al., 2018) which is persistently high without evidence of a decline since three decades (Mahmud et al., 2019) and the prevalence in rural areas and peri-urban areas is up to 25% (Umer and Iqbal, 2016). In Pakistan, genotype 3a is common (Haqqi et al., 2019) and to achieve WHO target of elimination of hepatitis C by 2030, treatment has to be provided to a million hepatitis C infected patients yearly (Altaf and Pasha, 2020).

Vitamin D receptors are hormonal receptors in the nucleus of the cell. They are ligand-activated regulatory proteins that direct the transcription machine to specific genomic sites to influence RNA production and therefore encoding proteins which are important for specific biological functions (Pike and Meyer, 2012). Vitamin D receptor is involved in different physiological processes...
and that include cell differentiation and proliferation and immune modulation (Adams and Hewison, 2008).

Several vitamin D receptor single nucleotide polymorphisms were found to be associated with the risk of hepatitis C infection (Wu et al., 2016), progression of the disease (Baur et al., 2012) as well as with the response to treatment (Garcia-Martin et al., 2013; Al-Aqmer et al., 2021). Baur et al. (2012) found Apal vitamin D receptor polymorphism to be inversely associated with the response to pegylated interferon with ribavirin and considered it as risk factor for failure of treatment. However, there were studies which reported no association between Apal vitamin D receptor polymorphism and the response to treatment (Arai et al., 2015; Abdelsalam et al., 2016; Wang et al., 2016; Thanapirom et al., 2019).

As no study was conducted specifically on hepatitis C genotype 3 patients receiving directly acting antiviral treatment, this study aimed to find out the association of Apal vitamin D receptor polymorphism with the response to directly acting antiviral treatment in hepatitis C genotype 3 Pakistani patients.

**MATERIALS AND METHODS**

This case control study was conducted after approval from the Ethical Review Board of Pakistan Kidney and Liver Institute, Lahore and included 66 responders who received hepatitis C antiviral treatment, daclatasvir and sofosbuvir (with ribavirin in case of cirrhotic patients), and achieved a sustained virologic response (HCV-RNA negative) three months after completing the treatment, males and females ≥ 18 years in age, and were matched in age and gender with 66 non-responders who received the same treatment and did not achieve a sustained virologic response (HCV-RNA positive) three months after completing the treatment. Patients with advanced liver disease, renal disease, hepatitis B, and HIV were excluded. After written consent was taken from the participants, demographic data and reports of hemoglobin, liver function tests, platelet count, prothrombin time, and INR were recorded. About 5 mL of blood was drawn for DNA extraction followed by polymerase chain reaction (PCR) of the DNA sequence containing the Apal restriction sites (rs7975232), restriction fragment length polymorphism analysis and gel electrophoresis.

Using the kit (Thermo Scientific #K0781), DNA extraction was done followed by PCR using the primers.

F 5’CAGAGCATGGCACAGGGAGCAA3’
R 5’GCAACTTCTCATGGCTAGGTCTC3’.

The 20 µL PCR reaction mixture contained 3 µL 25 mM MgCl₂, 2 µL 10X NH₄SO₄ buffer, 3 µL 2.5 mM dNTPs, 0.5 µL 5U/µL Taq polymerase, 5 µL DNA, 1.5 µL 10 µM forward primer, 1.5 µL 10 µM reverse primer, and 3.5 µL water. For the amplification of the DNA sequence containing the Apal restriction site (rs7975232), PCR underwent 35 cycles, each consisted of initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 65°C for 45 seconds, extension at 72°C for 45 seconds, and then final extension at 72°C for 10 minutes. The PCR product was of 744 base pairs.

Restriction fragment length polymorphism (RFLP) analysis for Apal was done using the enzyme Thermo Scientific Apal (#ER1411). The 30 µL mixture contained 10 µL PCR product (0.1-0.5 µg), 2 µL 10X buffer B, 2 µL Apal enzyme, and 16 µL nuclease free water. The mixture was incubated at 37°C for 8-16 h. Samples were run on 2% agarose gel and visualization was done under ultraviolet light and stored in the documentation system.

**Statistical analysis**

Data was analyzed using SPSS 24. T-test (for normally distributed data) and Mann-Whitney test (for not-normally distributed data) were used to compare age, BMI, hemoglobin, liver function tests, platelet count, prothrombin time and INR in cirrhotic and non-cirrhotic patients. Frequencies of Apal polymorphisms were studied in accord with the Hardy-Weinberg equilibrium. Chi-square test was used to assess the association of vitamin D polymorphisms with the response to treatment and with cirrhosis. Logistic regression was used to find out the association of Apal and other independent variables with the response to treatment. A p-value of < 0.05 was considered significant.

**RESULTS**

There were two groups, responders and non-responders, with 66 patients in each. There were 40 (63.6%) males and 26 (36.4%) females in each group and 33 (50%) cirrhotic and 33 (50%) non-cirrhotic patients in each group.

The restriction fragment length polymorphism analysis showed a single band of 744 base pairs in the AA wild homozygous genotype, three bands of 744, 527, and 217 base pairs in the Aa heterozygous genotype, and two bands of 527 and 217 base pairs in the aa homozygous mutant genotype (Fig. 1).

The frequencies of the AA, Aa, and aa Apal genotypes were 28 (42.4%), 27 (40.9%), and 11 (16.7%) in responders and 22 (33.3%), 26 (39.4%), and 18 (27.3%) in non-responders. The allelic distribution for the “A” and “a” alleles was 83 (62.9%) and 49 (37.1%) in responders and 70 (53%) and 62 (47%) in non-responders. Logistic regression showed Apal genotype “aa” as a significant predictor of treatment failure (p-value= .024, OR= 3.589, 95% CI= 1.181-10.911) (Table I).
Fig. 1. RFLP pattern of *ApaI* VDR polymorphism (rs7975232) in 2% agarose gel showing homozygous wild AA genotype with a band of 744 bp (Lanes# 6, 9, 10, and 11), heterozygous Aa genotype with the bands 744, 527, and 217 bp (Lanes# 3, 4, 7, and 8), and homozygous mutant aa genotypes with the bands 527 and 217 bp (Lanes# 1, 2, and 5).

Table I. Logistic regression of potential predictors of response to treatment in chronic hepatitis C genotype 3 patients.

| Variables                         | B       | p-value | OR (95% CI)               |
|-----------------------------------|---------|---------|---------------------------|
| AA                                | Referent|         |                           |
| aa                                | 1.278   | 0.024*  | 3.589 (1.181-10.911)      |
| Aa                                | 0.698   | 0.141   | 2.009 (0.794-5.082)       |
| Age                               | -0.032  | 0.238   | 0.969 (0.919-1.021)       |
| Gender                            | 0.200   | 0.655   | 1.221 (0.535-2.790)       |
| Smoking                           | -0.572  | 0.276   | 0.564 (0.202-1.579)       |
| BMI                               | 0.013   | 0.672   | 1.013 (0.955-1.074)       |
| Cirrhosis                         | 0.030   | 0.961   | 1.030 (0.309-3.433)       |
| Hemoglobin                        | -0.179  | 0.836   | (0.682-1.025)             |
| Platelets count                   | -0.003  | 0.223   | 0.997 (0.991-1.002)       |
| PT                                | -0.509  | 0.601   | 0.333-1.085               |
| INR                               | 0.897   | 0.602   | 2.453 (0.084-71.383)      |
| Total bilirubin                   | -0.211  | 0.825   | 0.810 (0.125-5.240)       |
| Direct bilirubin                  | 0.288   | 0.857   | 1.334 (0.058-30.832)      |
| AST                               | 0.004   | 0.789   | 1.004 (0.976-1.032)       |
| ALT                               | 0.009   | 0.420   | 1.009 (0.988-1.030)       |
| ALP                               | 0.003   | 0.756   | 1.003 (0.983-1.024)       |
| Serum albumin                     | -0.0404 | 0.336   | 0.668 (0.293-1.521)       |

* p-value < .05 is significant. OR, Odd’s ratio; CI, Confidence interval; BMI, Body mass index; PT, Prothrombin time; INR, International Normalized Ratio; AST, Aspartate aminotransferase; ALT, Alanine Aminotransferase; ALP, Alkaline phosphatase.

There was no association of *ApaI* VDR polymorphism with cirrhosis in responders and non-responders (Table II). The frequencies of *ApaI* VDR genotypes in males and females are shown in Table III and no significant difference was seen in the frequencies of *ApaI* VDR genotypes between males and females in both responder and non-responders.

Table II. Association of *ApaI* VDR polymorphism with cirrhosis.

| VDR genotypes | Responders (n=66) | Non-Responders (n=66) |
|----------------|-------------------|-----------------------|
|                | Cirrhotic (n=33)  | Non-cirrhotic (n=33)  |
| AA             | 15                | 13                    |
| aa             | 5                 | 6                     |
| Aa             | 13                | 14                    |

Chi square test 0.271, p-value 0.873 (0.576)

* p-value < .05 is significant. *Responders are patients who achieved sustained virologic response (SVR) three months after completing the treatment (HCV-RNA negative). *Non-Responders are patients who did not achieve sustained virologic response (SVR) three months after completing the treatment (HCV-RNA positive).

Table III. Distribution of *ApaI* VDR genotypes by gender.

| Apal genotype | Responders (n=66) | Non-Responders (n=66) |
|---------------|-------------------|-----------------------|
|                | Male n (%)        | Female n (%)          | Male n (%)        | Female n (%)          |
| AA             | 18 (45%)          | 10 (38.5%)            | 10 (25%)          | 12 (46.2%)            |
| aa             | 6 (15%)           | 5 (19.2%)             | 12 (30%)          | 6 (23.1%)             |
| Aa             | 16 (40%)          | 11 (42.3%)            | 18 (45%)          | 8 (30.8%)             |
| Total          | 40 (100%)         | 26 (100%)             | 40 (100%)         | 26 (100%)             |

Chi-Square test 0.349, p-value 0.840 (0.202)

* p-value < .05 is significant. For details of responders and non-responders, see Table II.

Significant differences were found in the levels of total bilirubin, direct bilirubin, ALP, serum albumin, PT, and INR between cirrhotic and non-cirrhotic patients in both the groups, responders and non-responders, and in the AST levels between cirrhotic and non-cirrhotic patients in the non-responders group (p-value < .05) (Tables IV and V).
Table IV. Age, BMI, platelets count, hemoglobin, LFTs, PT, and INR in cirrhotic and non-cirrhotic patients (in responders).

| Variable                  | Mean±SD / Mean rank | t / U | p-value |
|---------------------------|---------------------|-------|---------|
| Age (yrs)                 | 32.92±34.08         | 525.5 | 0.807   |
| BMI (kg/m²)               | 26.84±7.21          | -0.147| 0.883   |
| Platelets(×10³/µL)        | 35.11              | -1.313| 0.194   |
| Hb (g/dL)                 | 43.58              | -1.734| 0.088   |
| Total bilirubin (mg/dL)   | 44.71              | -2.621| 0.011*  |
| Direct bilirubin (mg/dL)  | 37.68              | -1.734| 0.088   |
| AST (U/L)                 | 64.79±23.73         | -1.99  | 0.843   |
| ALT (U/L)                 | 114.82±24.33        | -1.313| 0.194   |
| Serum albumin (g/dL)      | 41.17              | -1.734| 0.088   |
| PT (seconds)              | 49.15              | -2.621| 0.011*  |
| INR                       | 9.86               | -2.621| 0.011*  |

*p-value < .05 is significant. *T-test was used; *Mann-Whitney test was used. For other abbreviations, see Table I.

Table V. Age, BMI, platelets count, hemoglobin, LFTs, PT, and INR in cirrhotic and non-cirrhotic patients (in non-responders).

| Variable                  | Mean±SD / Mean rank | t / U | p-value |
|---------------------------|---------------------|-------|---------|
| Age (yrs)                 | 49.18±87.43         | -0.199| 0.843   |
| BMI (kg/m²)               | 35.70              | 0.178 | 0.859   |
| Platelets(×10³/µL)        | 37.59              | -0.199| 0.843   |
| Hb (g/dL)                 | 41.36              | -2.306| 0.024*  |
| Total bilirubin (mg/dL)   | 42.88              | -1.313| 0.194   |
| Direct bilirubin (mg/dL)  | 37.59              | -0.199| 0.843   |
| AST (U/L)                 | 64.85±23.43         | -2.306| 0.024*  |
| ALT (U/L)                 | 115.15±23.25        | -1.313| 0.194   |
| Serum albumin (g/dL)      | 26.35              | -1.313| 0.194   |
| PT (seconds)              | 42.73              | -2.306| 0.024*  |
| INR                       | 46.36              | -1.313| 0.194   |

For statistical details and abbreviations see Tables I and IV.

DISCUSSION

Our study found the mutant homogenous Apal genotype aa to be a risk factor and a predictor of treatment failure (OR= 3.589, 95% CI= 1.181-10.911). The mutant homogenous Apal aa genotype was also found to be a risk factor for treatment failure by Baur et al. (2012) (OR=2.67, 95% CI= 1.24-5.70). They found Apal polymorphism to be associated with failure to pegylated interferon and ribavirin treatment.

Contrary to our findings, Thanapirom et al. (2019) found no association between Apal polymorphism and the response to treatment; however, this could have been due to the presence of HCV genotypes 1, 2, 3, and 4 patients.
in their study whereas our study included only genotype 3 hepatitis C patients and the hepatitis C genotype could influence the response to treatment and might interact with the pharmacokinetics of the different drugs.

Wang et al. (2016) found no association between Apal VDR polymorphism and the response to treatment in HCV Chinese patients and similar results were reported by Abdelsalam et al. (2016) in genotype 4 Egyptian patients. In all these studies, the used antiviral treatment was interferon and ribavirin whereas in our study the treatment was directly acting antiviral treatment. The difference in the results could be due to pharmacogenetics, ethnicity, or the different HCV genotypes as the three factors do affect the response to treatment.

The association of Apal polymorphism with failure to treatment (daclatasvir and sofosbuvir with ribavirin in cirrhotic patient) could be explained in view of the effect of Apal polymorphism in forming a VDR protein that is less active and might cause a disturbance in the balance of T helper cell type 1/ T helper cell type 2, thereby causing diminished activity of the signaling pathways of vitamin D (Triantos et al., 2018).

This study is the first on the association of Apal polymorphisms with the response to directly acting antiviral treatment i.e., daclatasvir and sofosbuvir (with ribavirin in cirrhotic patient) in chronic hepatitis C genotype 3 patients. The previous studies were conducted on patients who received pegylated interferon and ribavirin whereas in our study the treatment was directly acting antiviral treatment. The difference in the results could be due to pharmacogenetics, ethnicity, or the different HCV genotypes as the three factors do affect the response to treatment.

The association of Apal polymorphism with failure to treatment (daclatasvir and sofosbuvir with ribavirin in cirrhotic patients) could be explained in view of the effect of Apal polymorphism in forming a VDR protein that is less active and might cause a disturbance in the balance of T helper cell type 1/ T helper cell type 2, thereby causing diminished activity of the signaling pathways of vitamin D (Triantos et al., 2018).

CONCLUSION

Apal VDR genotype aa is a predictor of failure to treatment in chronic hepatitis C genotype 3 patients and can be considered as a risk factor for treatment failure.

Conflict of interest

The authors have declared no conflict of interest.

Funding disclosure

None to declare

Disclaimer

None to declare.

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