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The Association of HLA Class II Alleles with the Response to Alfa-Interferon/Ribavirin Therapy in Chronic Hepatitis C Patients

[1]Awny Aly Abol-Ez Gawish, [1]Nahla Abd El-Hamid Mohammed, [1]Rasha Adel Sayed Ahmed Hussein and [2]Elsaid Galal El-Badrawy

[1]Department of Medical Microbiology and Immunology, Faculty of Medicine, Zagazig University, Egypt
[2]Department of Tropical medicine, Faculty of Medicine, Zagazig University, Egypt

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Corresponding Author:
Rasha Adel Sayed Ahmed Hussein
Department of Medical Microbiology and Immunology,
Faculty of Medicine,
Zagazig University, Egypt
Email: rahessin1981@gmail.com

Abstract: Human Leucocyte Antigens (HLA) class II appears to play an important role in the individual's immune response to viral infection. The present study was aimed at assessment of the relationship between HLA DRB1 alleles and the response to HCV combined therapy in Chronic Hepatitis C patients (CHC). We enrolled a total of 44 chronically infected HCV patients without Hepatitis B Virus (HBV) or Human Immunodeficiency Virus (HIV). Their mean age was 36.45±11.18 years (21-63). HLA-DRB1 typing was done by real time Polymerase Chain Reaction (PCR). ALT and Hemoglobin (Hb) levels were assessed as well as viral genotype was taken from patients' reports (HCV genotypes were 1, 2, 3 and 4 representing 13.6, 13.6, 4.5 and 68.18%, respectively). The most frequent alleles demonstrated among patients were DRB1*13 and DRB1*07 (31.8 and 36.4%, respectively). Analysis of DRB1 frequency between sustained responders and non responders revealed that DRB1*13 allele was significantly higher among sustained responders ($p<0.001$), while DRB1*07 allele was significantly higher among non responders ($p<0.01$). Female sex, HCV genotype 2 and pretreatment low serum HCV RNA level were associated with Sustained Virological Response (SVR). Also, elevated Alanine aminotransferase (ALT) level at the 1st week of therapy followed by return to baseline level at the 4th week and a decrease of the mean hemoglobin concentration at 4th week and 12th week of therapy were significantly associated with SVR. We concluded that the virological and special HLA patterns may possibly predict the response to combination therapy in CHC patients.

Keywords: Hepatitis C, Pegylated Interferon, Ribavirin, Human Leukocyte Antigen, Sustained Virological Response

Introduction

The World Health Organization (WHO) has declared hepatitis C a global health problem, with approximately 3% of the world's population infected with the Hepatitis C Virus (HCV). There are more than 170 million HCV chronic carriers at risk of developing liver cirrhosis and/or Hepatocellular Carcinoma (HCC) (Mosaad *et al*., 2010). Egypt has approximately 13% (about 10 millions) anti-HCV positive individuals mainly genotype 4a (Elkady *et al*., 2009).

Combination therapy with Pegylated Interferon-alpha (Peg-IFN) and ribavirin has been recommended and approved for patients with HCV infection (El Makhzangy *et al*., 2009). Although new era of treatment of hepatitis C are used in other countries, in Egypt under supervision of the National Committee for the Control of Viral Hepatitis (NCCVH) interferon still has been used plus ribavirin with added a new antiviral drug called Sofosbuvir to form triple therapy since October 2014 (NCCVH, 2014). So interferon and ribavirin are essential in treatment of hepatitis C

in Egypt. The goal of antiviral therapy is to achieve viral eradication. Clinically, therapeutic success is known as a Sustained Virologic Response (SVR), defined as undetectable serum HCV RNA for at least 6 months after cessation of therapy, thereby resolving liver inflammation and the progression to cirrhosis and hepatocellular carcinoma (Maylin *et al*., 2008). Treatment is costly and causes many side effects (Bhatti and Berenson, 2007). Also not all patients who receive antiviral therapy are able to clear the virus and respond to treatment; only about 55% of patients can successfully clear the virus depending on virological factors and host factors including immunogenetic factors (Dai *et al*., 2010).

The viral genotype, one of the most important viral predictors, has become the critical determinant of the length of combination therapy. On the other hand, some host factors such as age, gender, race, insulin resistance and host immune responses may also significantly affect drug response (Alberti and Benvegnù, 2003; Dai *et al*., 2009). Polymorphisms of the genes that regulate the immunity and the genes of Human Leukocyte Antigen (HLA) class I and II molecules are known to affect the clearance or persistence of viral antigens (Ocal *et al*., 2014). It was reported that HLA class II alleles, particularly DRB1 and DQB1 alleles, play a critical role in the outcome of HCV infection, influence susceptibility to or protection from HCV infection and also affect response to antiviral treatment (Shaker *et al*., 2013).

To our knowledge, HLA-associated alleles in Egyptian patients with hepatitis C have been studied using current methods include Sequence-Specific Oligonucleotide Probe Hybridization (SSOPH), Sequence-Specific Primers (SSP) and PCR-SSO luminex technology which are costly and require multiple steps (Shaker *et al*., 2013; Shaheen *et al*., 2013; Yosry *et al*., 2011).

In this study, we tried to assess the association between HLA class II (DRB1) alleles and response to combined interferon alpha and ribavirin therapy in HCV infected Egyptian patients, to investigate whether these alleles could be useful as predictors to treatment response, using a recent promising technology for HLA typing, (real time PCR) that has many advantages over other HLA typing methods. The primers for the allele-specific amplification and the internal control are mixed together in one well making this assay suitable for clinical use. This proposed method is fast, requires fewer handling steps, without further post-PCR processing for HLA genotyping and provides 100% sensitivity and specificity.

Materials and Methods

Study Population

A total of 44 Egyptian patients with confirmed Chronic Hepatitis C (CHC) treated with peg-IFN plus ribavirin were enrolled in this study (28 males, 16 females; mean ages 36.45±11.18 years; range from 21-63). None of the patients had received prior antiviral treatment. Before peg-IFN therapy, diagnosis of CHC was established on persistent (>6 months) elevation of Alanine aminotransferase (ALT) concentration 1.5 times above the upper limit of normal, positive results of antibody to HCV testing, positive HCV RNA testing in serum and histological evidence of chronic hepatitis and earlier fibrosis stages (Metavir stage ≤F2). The Metavir scoring system described as follows: For activity, A0 is no histological activity, A1 mild activity, A2 moderate activity and A3 severe activity; for fibrosis, F0 is no fibrosis, F1 portal tract expansion by fibrosis, F2 less than 50% bridging fibrosis, F3 more than 50% bridging fibrosis without cirrhosis and F4 established cirrhosis (Shaker *et al*., 2013). They were followed from March 2012 to March 2014 at outpatient clinics of hepatology unit, Al-Ahrar Hospital (Zagazig, Egypt) under the supervision of the National Committee for the Control of Viral Hepatitis (NCCVH). Patients were negative for Hepatitis B surface Antigen (HBsAg), autoimmune hepatitis, antinuclear antibodies, Human Immunodeficiency Virus (HIV) and active schistosomiasis. HCV genotypes of the patients were taken from the patients´ records.

The study protocol and informed consent were approved by the Ethical Committee of NCCVH.

Study Design

All patients were treated with subcutaneous injections of peg-IFNα-2a (180 µg/week) combined with orally given ribavirin (1000-1200 mg/day, body weight based) for 48 weeks. The presence of HCV RNA in the serum was tested at weeks 12, 24, 48 and 72 by real-time PCR. Patients achieving SVR in terms of clearance of serum HCV RNA by real time PCR at the end of the therapy and for 6 months after the cessation of therapy were grouped as sustained responders (group 1). All other patients did not achieve SVR were classified as non-responders (group 2) (Dai *et al*., 2010).

Real-Time PCR for Serum HCV RNA

Serum HCV RNA was determined by standardized automated quantitative Polymerase Chain Reaction (PCR) (COBAS AmpliPrep/COBAS TaqMan HCV Test, Roche molecular system, USA). All steps were

performed automatically including; sample preparation to isolate HCV RNA, reverse transcription of the target RNA to generate complementary DNA (cDNA) then automated simultaneous PCR amplification and detection of cleaved dual-labeled oligonucleotide detection probe specific to the target. Serum HCV RNA was expressed as International Units (IU) per ml. The detection limit was from 15 to $6.90\chi 10^7$ IU/ml.

HLA DRB1 Typing

From both groups genomic DNA was extracted and purified from whole blood using PREP-RAPID GENETICS DNA Extraction Kit (DNA technology, Russia), according to the manufacturer's instructions. The supernatant containing isolated DNA was stored at -20°C to be used for PCR. Genotyping of HLA class II alleles were performed at DRB1 regions using real-time PCR (DNA-Technology, Russia) which applies DT*lite4* real-time thermal cycler, according to the manufacturer's instructions. Paraffin sealed PCR-mix A and B strips, included in the kit, contained primers, a fluorogenic locus-specific probe, master mix and internal control which were pre-aliquoted and frozen in the strips so that only Taq-polymerase solution and extracted DNA were added.

Statistical Analysis

All patients' data were tabulated and processed using Statistical Package for Social Sciences (SPSS) software 16.0 for Windows XP. Qualitative data were expressed in number and percent; they were compared using Chi-square test or Fischer's exact test when appropriate. Quantitative data were expressed in terms of mean and standard deviation. They were compared using Student's t-test. Odds Ratios (ORs) were calculated for different alleles, whenever a significant P value in the distribution of a specific allele was observed. In all tests, p value less than 0.05 was considered to be significant.

Results

Among the 44 CHC patients, the mean pretreatment HCV RNA level was 575640±360260 IU/ml, baseline ALT was minimally elevated (mean 1-1.5 times the Upper Limit of Normal (ULN) level) in 50% and moderately elevated (mean >1.5 times ULN) in the other 50% of the patients. Thirty patients were infected by genotype 4 (68.18%) while other genotypes 1, 2 and 3 were present in 13.6, 13.6 and 4.5% of CHC patients respectively (Table 1).

Response to Combination Therapy with PEG-IFN 2a and Ribavirin in CHC Patients

After PEG-INF/ribavirin therapy, 40.9% (18/44) of patients achieved SVR and 59.1% (26/44) were non responders. Comparison of the clinical, virological and laboratory factors between sustained responders and non-responders is shown in Table 1. The univariate analysis of clinical and virological factors showed that female sex, genotype 2, lower pretreatment HCV RNA level, higher ALT at 1stweek of therapy and lower Hb level at 4th week and 12th week were associated with a higher probability of sustained response with statistically significant difference for each ($p<0.05$). Ribavirin dose reduction was necessary in these HCV treatment related anaemic patients.

HLA DRB1 Alleles and Response to Therapy in CHC Patients

HLA DRB1 typing was performed in all patients (Table 2). It was demonstrated that HLA DRB1 *07 and HLA DRB1 *13 were the most frequent alleles observed in CHC patients representing 31.8 and 36.4% respectively. The frequency of HLA DRB1 alleles in the sustained responders and non-responders were compared (Table 3). In univariate analysis, an increase in frequency ($p<0.001$) of HLA DRB1 *13 was observed in sustained responders (77.8%; 95% confidence interval with an Odd Ratio (OR) of 42 (2.38-77.29)). On the other hand, DRB1∗7 was significantly ($p<0.01$) more frequent in non responders (53.8%).

HLA DRB1 Alleles and Response to Therapy among CHC Patients with HCV Genotype 4

Among the18 patients with sustained response only 8 had HCV genotype 4 (44.5%) whereas in non responders HCV genotype 4 was observed in 22 of 26 patients (84.6%). Comparison of clinical, virological features, HLA DRB1*07 and *13 frequency in patients infected by HCV genotype 4 according to response to therapy is shown in Table 4. Eight of the 22 CHC patients with no response and HCV genotype 4 had the DRB1*07 allele (36.4%) with its absence in sustained responders with HCV genotype 4 ($p<0.01$). On the other hand 6 of 8 patients with sustained response and HCV genotype 4 had the DRB1 *13 allele (75%) ($p<0.001$) with its absence in non responders.

In HCV genotype 4, like other HCV genotypes infected patients, easy fatigability was less frequent in sustained responders than non responders and female gender respond to therapy more frequently than male ($p<0.05$). In addition, in patients infected with HCV genotype 4, pretreatment serum HCV RNA level was significantly lower ($p<0.05$) in the eight patients with sustained response as compared to 22 patients with no response.

Table 1. The Clinical, virological and laboratory features of 44 CHC patients according to response to therapy

	Group I Sustained responders (n = 18)	Group II Non responders (n = 26)
Male/Female	6/12*	22/4
Mean age (years ± SD)	30.22±5.19	40.77±12.30
Symptoms (%)		
No	16 (88.9%)*	8 (30.8%)
Easy fatigability	2 (11.1%)*	10 (38.5%)
Abdominal pain	0 (0%)	6 (23.1%)
Jaundice	0 (0%)	2 (7.7%)
HB*(gm/dl):*		
Base line Mean ± SD	13.94±1.72	14.39±1.67
4weeks Mean ± SD	11.96±1.98*	13.82±1.75
12weeks Mean ± SD	8.94±1.87**	13.40±1.92
ALT(U/L):		
Baseline: (%)		
Normal	0 (0%)	0 (0)
Minimal elevated a[1]	8 (44.4%)	14 (53.8%)
Moderately elevated a[2]	10 (55.6%)	12 (46.2%)
1[ST] *week: (%)*		
Normal	4 (22.2%)*	8(30.8%)
Minimal elevated	0 (0%)*	10 (38.5%)
Moderately elevated	14 (77.8%)*	8 (30.8%)
4[th]*week: (%)*		
Normal	16 (88.9%)	20 (76.9%)
Minimal elevated	2(11.1%)	2 (7.7%)
Moderately elevated	0 (0%)	4(15.4%)
Genotype (N)		
1 (6)	2 (11.1%)	4(15.4%)
2 (6)	6 (33.3%)*	0 (0%)
3 (2)	2 (11.1%)	0 (0%)
4 (30)	8 (44.5%)*	22 (84.6%)
Viral load:		
(IU/ml)		
Base line Mean ± SD	273780±269545**	784620±251085

*p<0.05 significant

**p<0.001 highly significant

a[1]minimally elevated ALT level means 1-1.5 times the Upper Limit Of Normal (ULN) level (ULN = 33 U/L for males and 24 U/L for females

a[2]moderately elevated mean >1.5 times ULN.

Table 2. HLA class II (HLA DRB1) alleles of the all CHC patients

	CHC patients (*n* = 44)	
Variable	No	%
Alleles:		
*DRB1*01*	6	13.6
*DRB1*03*	4	9.1
*DRB1*04*	2	4.5
*DRB1*07*	14	31.8
*DRB1*08*	4	9.1
*DRB1*10*	8	18.2
*DRB1*11*	10	22.7
*DRB1*12*	2	4.5
*DRB1*13*	16	36.4
*DRB1*14*	2	4.5
*DRB1*15*	6	13.6

Table 3. Distribution of HLA DRB1 alleles and response to therapy in CHC patients

	Group I (Sustained responder) (n=18)		Group II (Non-responder) (n=26)				
	No	%	No	%	χ^2	p	OR
Alleles:							
*DRB1*01*	4	22.2	2	7.7	0.95	0.33 N.S	3.43 (0.18-17.72)
*DRB1*03*	2	11.1	2	7.7	0.08	0.78 N.S	1.50 (0.01-6.30)
*DRB1*04*	2	11.1	0	0.0	1.51	0.22 N.S	------
*DRB1*07*	0	0.0	14	53.8	7.11	0.01*	--------
*DRB1*08*	2	11.1	2	7.7	0.08	0.78 N.S	1.50 (0.01-6.30)
*DRB1*10*	2	11.1	6	23.1	0.51	0.47 N.S	0.42 (0.01-6.39)
*DRB1*11*	2	11.1	8	30.8	1.17	0.28 N.S	0.28 (0.01-3.93)
*DRB1*12*	0	0.0	2	7.7	0.73	0.39 N.S	-------
*DRB1*13*	14	77.8	2	7.7	11.29	0.001**	42 (2.38-77.29)
*DRB1*14*	0	0.0	2	7.7	0.73	0.39 N.S	------
*DRB1*15*	2	11.1	4	15.4	0.08	0.77 N.S	0.69 (0.15-12.98)

OR: Odds Ratio
*p<0.05 significant
**p<0.001 highly significant

Table 4. Comparison of clinical, virological features, HLA DRB1*07 and *13 frequency in patients infected by HCV genotype 4 according to response to therapy

	Genotype 4 Sustained responders (n = 8)	Genotype 4 Non responders (n = 22)
Male/Female	2/6**	18/4
Mean age (years ± SD)	34.25±3.36	39.82±12.37
Symptoms (%)		
No	6 (75%)	8(36.4%)
Easy fatigability	2 (25%)*	10 (45.5%)
Abdominal pain	0 (0%)	4 (18.1%)
Jaundice	0 (0%)	0(0%)
HLA DRB1 type:		
HLA DRB1*07	0 (0%)**	8 (36.4%)
HLA DRB1* 13	6 (75%)**	0(0%)
Viral load:		
Base line Mean ± SD	369750±390188*	690910±320732

*p<0.05 significant
**p<0.001 highly significant

Discussion

IFN-a is an effective therapy for CHC but only about 55% of patients can successfully clear the virus depending on virological factors and host factors including immunogenetic factors (Dai *et al*., 2010).

HLA class II genes are a crucial factor that regulates immune response through the presentation of viral antigens to CD4 T lymphocytes (Shiina *et al*., 2009). HLA alleles are highly polymorphic among different populations which give variation in immune response (Timm *et al*., 2007).

In the present study we investigated the possible association between HLA class II DRB1 alleles and response to PEG-IFN/ribavirin therapy in patients with CHC.

In the present study, HLA DRB1*07 and HLA DRB1*13 representing 31.8 and 36.4% of CHC patients respectively were the most prevalent alleles demonstrated. By comparing the frequencies of HLA DRB1 alleles between patients who responded and those who did not respond to combination treatment, HLA DRB1 *13 allele showed a significant association with SVR while HLA DRB1 *07 allele showed a significant association with non responded patients.

Regarding the association of HLA DRB1 alleles and response to combination treatment, CHC patients with genotype 4 showed the same result.

Effective presentation of viral antigens to CD4+ T cells by HLA class II molecules is the key regulation of optimum immune response against viral infection. With the upregulated expression of immunogenetic molecules which enhances the immune response by IFN, the genetic variations at HLA loci with respect to antigen presentation might be a candidate related to response to IFN based therapy.

The association of HLA alleles with HCV infection in the Egyptian population has been addressed in several studies. Our result concerning HLA DRB1*13 is in

agreement with Shaker *et al*. (2013) who reported that DRB1∗1301, DRB1∗1361, DRB1∗ 1369, DRB1∗13 alleles to be significantly more frequent in responders than in non responders. Concerning our results of HLA DRB1 *07, we agree with Zekri *et al*. (2005) who showed that HLA class II allele DR* 07 was significantly encountered in HCV positive family members than negative members. Furthermore, our results are concordant with those of Hendy *et al*. (2011), who demonstrated DRB1*7 and DQB1*02 alleles to be associated with viral persistence.

These findings were also supported by a cohort study on Pakistani HCV patients treated with IFN therapy which reported that HLA-DRB1*07 individually or in combination with HLA-DQB1*02 was associated with viral persistence, however unlike our results, HLA-DRB1*11 was found to be associated with viral clearance (Ali *et al*., 2010). In addition, in the Polish population, HLA DRB1*07 and DRB1*13 were more frequent in non-responders and responders to treatment respectively, but without statistical significance (Piekarska *et al*., 2002).

A great disagreement is noticed with Dincer *et al*. (2001) who demonstrated that patients not responding to treatment with interferon more frequently revealed the presence of DRB1*13 allele than among others.

In general, different studies in different populations had reported presence of different specific HLA alleles or significant haplotype to be correlated with response or no response to treatment. In Taiwan, HLA-DRB1*15 allele was positively correlated with a sustained response to IFN-α (Bruno *et al*., 2004). In Caucasian patients from Canada, DRB1∗0404 allele was associated with response to treatment (De Re *et al*., 2010). In Turkish patients infected with HCV, HLA DRB1 *10 allele was associated with viral clearance (Ocal *et al*., 2014).

Meanwhile, other studies found no difference in distribution of HLA alleles between responders and non responders to antiviral therapy, for instance, in German, Knolle *et al*. (1998) have reported that, in patients suffering from chronic hepatitis C and treated with IFN-α, the pretreatment viral factors, not host factors, were significantly correlated with treatment response. Romero-Gomez *et al*. (2003) reported that in Spanish population, HLA class II showed no effect on response to interferon treatment.

Although our results coincided with other studies made in Egypt regarding association of HLA DRB1 *13 and HLA DRB1*07 with response to combined treatment of pegylated interferon and ribavirin, they were different from other studies in other populations which may due to the difference in the race, genetic background of the patients and treatment response.

Several pretreatment related features as younger age, female sex, in addition to clinical parameters including non-genotype 1 infection and lower levels of viremia have been currently reported to be associated with a better response (Dai *et al*., 2010).

In accordance with these previous reports, our study found that younger age; female sex, non-1 HCV genotype, particularly genotype 2 and low serum HCV RNA were associated with a sustained response.

Although HCV genotyping is an important predictor for the treatment response, no patient should be left without therapy on the basis of the genotype alone because the predictive value of HCV genotyping for interferon based therapy is only 55% (Imran *et al*., 2013).

Complications of interferon/ribavirin therapy are so many. Anemia can be one of the most clinically significant side effects of therapy. Anemia is a dose-dependent side-effect of ribavirin administration. The mean Hb concentration was significantly different in two groups. A decrease of the mean hemoglobin concentration at 4^{th} week and 12^{th} week of therapy significantly associated with sustained virological response. Our results are supported by that of the results of Urbanek *et al*. (2013) who concluded that the decrease in Hb concentration may serve as an additional predictive factor of SVR in patients with chronic HCV infection under antiviral treatment and the most useful factors are a Hb decrease at 4^{th} week and 12^{th} week of therapy.

Among the most sensitive and widely used liver enzymes are the aminotransferases. ALT acts as a biomarker of hepatocyte injury and is associated with the progression of hepatic fibrosis (Ribeiro *et al*., 2003).

Most of responders in our study (77.8%) showed a moderate elevation of ALT level at 1^{st} week of therapy when compared to its pretreatment value, followed by return to baseline or within normal ranges at the 4^{th} week. The elevated ALT level during therapy may be attributed to degenerative, apoptotic and necrotic changes in hepatocytes, probably a result of the cytotoxic effects of IFN, rather than to increased hepatitis activity (Fujimori *et al*., 2002).

Our result is in agreement with Ribeiro *et al*. (2003) who observed 4 patterns of ALT change during therapy; one of them was an initial increase of ALT level followed by a decrease in the long term therapy in approximately 50% of the studied patients. In most patients, this behavior was accompanied by a viral load drop of more than 1 log from baseline over the same period.

Conclusion

We are aware with the main limitation of this study, which is the relatively small number of cases because of the high cost of the test.

However we can conclude that certain HLA-DRB1 alleles may act as predictors for response to treatment in the Egyptian population e.g. HLA DRB1 *07 and HLA

DRB1 *13. This is important for disease management and deciding which patient would most likely benefit from IFN therapy and which would not.

Recommendation

In Egypt, there is still a struggle for approving the new direct-acting inhibitors of HCV that will be used in combination with interferon or without the application of interferon, so further future studies of factors that may predict the treatment outcome of the new combinational therapies are required. HLA typing with the same combination of therapy with added Sofosbuvir as a new strategy started recently in Egypt.

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Author's Contributions

Awny Aly Abol-Ez Gawish: Designed the research plan and organized the study.

Nahla Abd El-Hamid Mohammed and Dr.Rasha Adel Sayed Ahmed Hussein: Participated in all experiments, coordinated the data-analysis and contributed to the writing of the manuscript.

Elsaid Galal El-Badrawy: Participated in proper selection of the study cases, collection of clinical samples, clinical data, revision of the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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