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Determination of Non-Organ Specific Autoantibodies in Patients with Chronic Hepatitis C and Association with HLA DRB1 (*04) Allele

(Penentuan Autoantibodi Khusus Bukan Organ pada Pesakit Hepatitis C Kronik dan Perkaitan dengan Alel HLA DRB1 (*04))

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ABSTRACT

The regulation of immune mechanisms is controlled by major histocompatibility complex/human leukocyte antigen (MHC/HLA). Polymorphisms of the HLA region have an impact on susceptibility to complex infectious and autoimmune diseases. The present study was carried out to determine the frequencies of ASMA, AMA, ANA, dsDNA, and anti-LKM-1 auto-antibodies in hepatitis C patients and to determine their association with the HLA DRβ1 (*04) locus. It was a cross-sectional, analytical study, and 86 patients with chronic HCV were recruited. The presence of auto-antibodies (ASMA, AMA, ANA, dsDNA, and anti-LKM-1) was determined by indirect immunofluorescence and ELISA, while the HLA DRβ1 (*04) allele was assessed by sequence-specific conventional PCR. ANA was detected in 41%, ASMA in 17.4%, AMA in 7%, LKM-1 in 5.8% dsDNA in 4.6% of CHC patients while HLA-DRβ1 (*04) was present in 3.5% of patients, but this was not significantly associated with these auto-antibodies.

Keywords: Auto-antibodies; HCV; hepatitis C; HLA DRβ1; immunofluorescence

ABSTRAK

Pengawalaturan mekanisme imun dikawal oleh kompleks histoserasian utama/antigen leukosit manusia (MHC/HLA). Polimorfisme rantau HLA mempunyai kesan ke atas kerentanan kepada penyakit berjangkit dan autoimun yang kompleks. Kajian ini dijalankan untuk menentukan kekerapan ASMA, AMA, ANA, dsDNA dan anti-LKM-1 auto-antibodi pada pesakit hepatitis C dan untuk menentukan hubungan mereka dengan lokus HLA DRβ1 (*04). Ia adalah kajian keratan rentas, analisis dan 86 pesakit dengan HCV kronik telah diambil. Kehadiran auto-antibodi (ASMA, AMA, ANA, dsDNA dan anti-LKM-1) ditentukan oleh imunofluoresensi tidak langsung dan ELISA, manakala alel HLA DRβ1 (* 04) dinilai oleh urutan khusus PCR konvensional. ANA dikesan pada 41%, ASMA pada 17.4%, AMA pada 7%, LKM-1 dalam 5.8% dsDNA pada 4.6% pesakit CHC manakala HLA-DRβ1 (*04) hadir pada 3.5% pesakit, tetapi ini tidak secara signifikan dikaitkan dengan auto-antibodi ini.

Kata kunci: Auto-antibodi; HCV; hepatitis C; HLA DRβ1; imunopendafluor

Introduction

Hepatitis C is an inflammatory condition of the liver that is triggered by the hepatitis C virus (HCV), which belongs to the family Flaviviridae. It is an enveloped virus with a single-stranded RNA genome with a positive polarity that can cause both acute and chronic inflammation of the liver (Rosen & Hugo 2011). Around the world, there are about 0.2 billion carriers of HCV (Craxì et al. 2008). In Pakistan, 8.64% population is infected with HCV (Arshad & Ashfaq 2017). Antibodies against HCV recognize and clear the invading organism (Bogdanos, Mieli-Vergani & Vergani 2005). However, autoantibodies are produced when the immune mechanism is not functioning properly (Shoenfeld et al. 2013).

Normally, autoreactive B cells are deleted by the tolerance mechanism, but chronic infections may lead to a breach in tolerance (Metwally et al. 2012). In some CHC patients, tolerance is deranged by infection, so the risk of autoimmune antibodies is increased (Roughan et al. 2012). Development of certain non-organ specific autoantibodies (NOSAs) occurs during CHC infection as a consequence of a break-in tolerance (Bogdanos, Mieli-Vergani & Vergani 2005), e.g., anti-smooth muscle antibody (ASMA), anti-nuclear antibodies (ANA) and anti-liver/kidney microsomal-1 (LKM-1) antibodies (Himoto & Nishioka 2013). Another nuclear antibody that recognizes and binds to DNA is the anti-dsDNA antibody (Pisetsky 2016). The severity of inflammation and fibrosis, and liver profile markers such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and IgG levels in HCV infected patients are correlated with NOSAs (Chrétien et al. 2009). In humans, the regulation of immune mechanisms is controlled by major histocompatibility complex/human leukocyte antigen (MHC/HLA). Peptides from inside cells are presented by MHC class I (A, B, and C). HLAs corresponding to MHC class II (DP, DQ, and DR) present antigens to T-lymphocytes (Janeway Jr. et al. 2001). Polymorphisms in the HLA region have an impact on an individual's susceptibility to complex infectious and autoimmune diseases (Hill et al. 2011; Song et al. 2016). Antibody-mediated immune responses against HLA class II molecules are involved in the acceleration or pathogenesis of liver injury (Yamagiwa et al. 2014). The absence of HLA-DR3 or HLA-DR4, HLA-DR13 alleles has been suggested to be a risk factor for autoimmune hepatitis (Czaja et al. 2006). In the Pakistani population, a strong association has been reported between autoimmune hepatitis (AIH) and HLA-A2, HLA-A9, HLA-β15, HLA-B40, and HLA-DR6 with its subtypes HLA-DRβ1⁄13 and HLA-DRβ1⁄14 (Hassan et al. 2013). At the time of infection, the T cell reaction is linked with viral clearance, with one study suggesting that DR11 may present HCV epitopes to CD4 cells more efficiently than other alleles. Moreover, in patients with sustained virus infection, DRB1*11 may be critical in limiting the spread of the virus so that liver injury remains minimal. In DRB1*11 negative patients, continuous liver damage is mostly caused by non-specific bystander inflammatory processes that may accelerate the development of fibrosis (Renou et al. 2002). Regarding HCV clearance or persistence, five HLA class I allele groups (B*18, B*27, B*57, Cw*01, and Cw*04) are associated (Kuniholm et al. 2010). In HCV, the protective alleles of HLA-B*27 present the most conserved epitopes of HCV to elicit potent cytotoxic T cell responses, thereby, minimising the ability of HCV to escape from host immune responses (Rao et al. 2015). Although the implicated genes differ, the HLA class II-restricted immune reaction is critical regarding HCV disease outcome (Thio et al. 2002). The timely recognition of genetic factors may be helpful in anticipating disease outcome, setting guidelines for the appropriate therapy for patients with a poor prognosis and in assuring the development of new curative strategies (Scotto et al. 2003). The aim of the present study was to determine the frequencies of ASMA, ANA, AMA, anti-dsDNA and anti-LKM-1 auto-antibodies in CHC patients and to determine their association with HLA allele DRβ1 (*04) locus.

Materials and Methods

Study population

All (86) HCV infected patients were recruited from the Department of Gastroenterology and Hepatology, Sheikh Zayed Hospital, Lahore, from March to November 2018. The study was approved by the Ethical Review Committee of the University of Health Sciences Lahore (No. UHS/Education/126-18/423). Written informed consent was obtained with all the relevant details such as age, gender, clinical findings, duration of disease, and therapeutic history in a structured questionnaire. Approximately 5 mL of blood was collected for immunofluorescence, ELISA, and DNA extraction. The study subjects were between 18-65 years of age of either gender, diagnosed with HCV (ELISA and PCR positive) of more than 6 months duration, and either treatment naïve or on no treatment. Patients with HBV or other viral infections, liver disease, malignancy, i.e., HCC, or autoimmune disease (e.g., SLE and RA) were excluded.

Immunofluorescence and other assays

ASMA, AMA, and LKM-1 antibodies in the serum were detected by indirect immunofluorescence using rat kidney, liver, and stomach slides (Euroimmun FA 1710-1010, Germany), while dsDNA and ANA in the serum by ELISA as per the manufacturer's instructions (Inova Diagnostics, USA). Genomic DNA was extracted from whole blood using the phenol-chloroform method (Barker 1998). [22] and HLA DRβ1 (*04) allele with the primer sequence F (5' -TTG TGG CAG CTT AAG TTT GAA T- 3'), R (5' -CCG CCT CTG CTC CAG GAG- 3') was detected by sequence-specific conventional PCR.

Thermal cycling was performed on an iCycler (Bio-Rad, USA), where the DNA was denatured at 95 ℃ for 4 min, followed by 30 cycles of denaturation at 94 ℃ for 30 s, annealing at 65 ℃ for 30 s and extension at 72 ℃ for 30 s, followed by 7 min final extension at 72 ℃ and the product was stored at 4 ℃. The amplified PCR product was electrophoresed on a 2% agarose gel (Vivantis, Malaysia).

STATISTICAL ANALYSIS

Analysis of the data was performed using the software package SPSS (Statistical Package for Social Science) version 20. Mean ± SD was calculated for quantitative variables, such as age and biochemical tests of the liver. Frequencies were calculated for qualitative variables, such as gender. Data were analyzed using the chi-squared (χ^2) test. Frequencies of autoantibodies and the HLA-DRβ1 (*04) allele were compared using the chi-squared test. Odds ratios (OR) and confidence intervals (CIs) were calculated using logistic regression. p-value ≤ 0.05 was considered statistically significant.

RESULTS

A total of 86 diagnosed chronic HCV patients were recruited. The details regarding the age of the patients and their anti-HCV treatment status are given in Table 1.

TABLE 1. Demographic data of chronic HCV patients

Mean age (years)		43.47
Gender n (%)	Female	45 (52.4)
	Male	41 (47.6)
Without anti HCV treatment n (%)		44 (51.2)
Anti HCV treatment n (%)		42 (48.8)

%= Percentage, n = Number of patients

The biochemical profile of patients receiving anti-HCV treatment and without treatment is given in Table 2. Levels of alkaline phosphatase (ALP), alanine transferase (ALT), aspartate transferase (AST), albumin, and bilirubin were high in patients without treatment as compared to those receiving anti-HCV therapies; the comparison for ALP was statistically significant (Table 2).

TABLE 2. Comparison of laboratory tests between two study groups in the chronic hepatitis C patients

Variable (Mean ± SD)	Study groups		p-value
	On treatment (n=42)	Without treatment (n=44)	
ALP	(110.1 ± 41.89)	(197 ± 142.24)	0.006*
ALT	(48.70 ± 34.92)	(64.63 ± 76.7)	0.323
AST	(43.88 ± 27.23)	(48.96 ± 46.22)	0.621
Albumin	(5.55 ± 6.83)	(4.58 ± 1.95)	0.483
Bilirubin	(0.59 ± 0.26)	(0.73 ± 0.54)	0.229

n = Number of patients, *p≤0.05=statistically significant

AMA, ASMA, and anti-LKM-1 were present in 7%, 17.4%, and 5.8%, while ANA and anti-dsDNA were found in 41% and 4.6% of CHC patients, respectively (Figures 1-4).

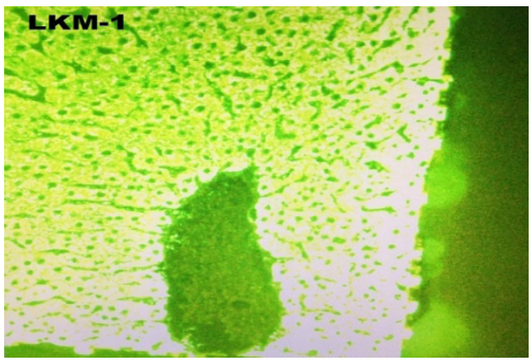

FIGURE 1. Fluorescence of LKM-1 of frozen rat liver section

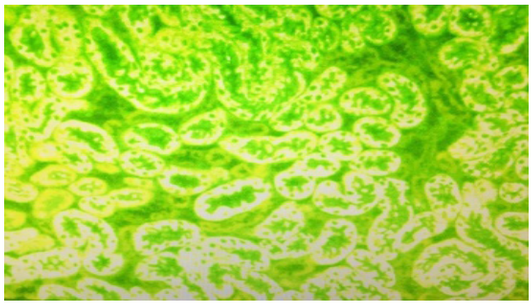

FIGURE 2. Fluorescence of LKM-1 of frozen rat kidney section

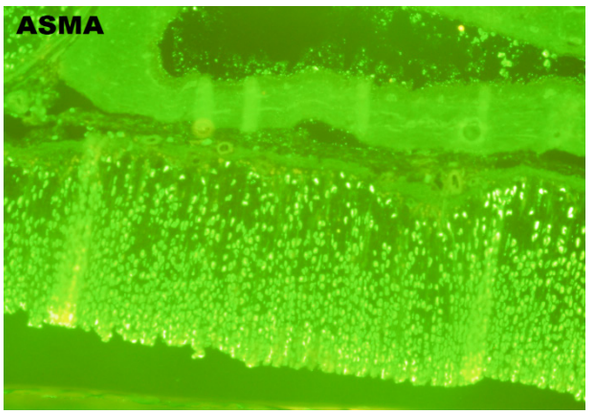

FIGURE 3. Fluorescence of smooth muscles of frozen rat stomach and intestinal section

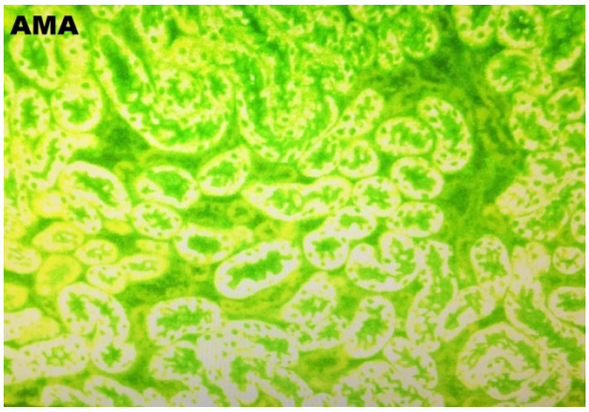

FIGURE 4. Fluorescence AMA of frozen rat kidney section

HLA DRβ1 (*04) allele was present in 3.5% of patients. None of the patients had HLA DRβ1 (*04), ASMA, or LKM-1. Only one patient had HLA DRβ1 (*04) and AMA with an odds ratio of 7.80, and this was not statistically significant (p=0.197). These results suggest no statistically significant association between these autoantibodies and the HLA DRβ1 (*04) allele in CHC patients (Tables 3 & 4).

TABLE 3. Association of ASMA, LKM-1, and AMA autoantibodies with HLA-DRβ1 (*04) allele in chronic hepatitis C patients

Variables		HLA DRβ1 (*04)		Odds ratio (Confidence interval)	p-value
	Positive	Negative			
ASMA	Positive	0	15	1.04 (0.994 -1.096)	1.00
	Negative	3	68		
LKM-1	Positive	0	5	1.03 (0.995- 1.084)	1.00
	Negative	3	78		
AMA	Positive	1	5	7.80 (0.600-101.41)	0.197
	Negative	3	77		

%= Percentage, ASMA =Anti-smooth muscle autoantibody, LKM-1 =Liver kidney microsomal type-1 autoantibody, AMA =Anti-mitochondrial antibody, *$p \leq 0.05$=statistically significant

TABLE 4. Association of ANA and dsDNA autoantibodies with HLA-DRβ1 (*04) allele in chronic hepatitis C patients

Variables		HLA DRβ1 (*04) (n)	
		Positive	Negative
ANA	Positive	0	28
	Negative	3	55
dsDNA	Positive	0	8
	Negative	3	75

n= Number of patients, ANA =Anti-nuclear antibody, dsDNA =Anti- double stranded DNA antibody

The serum level of autoantibodies was also compared in males and females, with the number of males and females having autoantibodies, their odds ratios, confidence intervals, and a comparison between genders given in Table 5.

TABLE 5. Comparison of autoantibodies between the two genders in the chronic hepatitis C patients

		Male	Female	Odds ratio (95% Confidence interval)	*p* value
ASMA	Positive	7	8	0.95 (.312 -2.907)	1.00
	Negative	34	37		
LKM-1	Positive	3	2	1.69 (0.269-10.705)	0.66
	Negative	38	43		
AMA	Positive	3	3	1.10 (.210-5.809)	1.00
	Negative	38	42		
ANA	Positive	15	21	0.65 (0.27-1.56)	0.38
	Negative	26	24		
dsDNA	Positive	1	3	0.35 (0.03-3.50)	0.61
	Negative	40	42		

%= Percentage, ASMA =Anti-smooth muscle autoantibody, LKM-1 =Liver kidney microsomal type-1 autoantibody, AMA =Anti-mitochondrial antibody, ANA =Anti-nuclear antibody, dsDNA =Anti- double stranded DNA antibody *$p \leq 0.05$=statistically significant

Auto-antibody production was compared with the duration of infection (Table 6). ASMA, LKM-1, and ANA were present in patients with an infection duration of fewer than five years. The presence of dsDNA in the studied subjects was similar, while AMA was present in patients with an infection duration of more than five years, but this was not statistically significant.

TABLE 6. Comparison of autoantibodies with the duration of disease in chronic hepatitis C patients

Variables		Duration of infection		Odds ratio (95% Confidence interval)	*p*-value
	< 5 years	> 5 years			
ASMA	Positive	11	4	1.23 (0.35 -4.31)	0.77
	Negative	49	22		
LKM-1	Positive	3	2	0.63 (0.99-4.02)	0.63
	Negative	57	24		
AMA	Positive	2	4	0.19 (0.03-1.11)	0.06
	Negative	58	22		
ANA	Positive	22	14	0.49 (0.19-1.26)	0.15
	Negative	38	12		
dsDNA	Positive	2	2	0.41 (0.55-3.11)	0.58
	Negative	58	24		

%= Percentage, ASMA =Anti-smooth muscle autoantibody, LKM-1 =Liver kidney microsomal type-1 autoantibody, AMA =Anti-mitochondrial antibody, ANA =Anti-nuclear antibody, dsDNA =Anti- double stranded DNA antibody *$p \leq 0.05$=statistically significant

DISCUSSION

Hepatitis C is an inflammatory condition of the liver. It has been implicated in both acute and chronic hepatitis infection. Under certain pathological conditions, the immune response is targeted against the self, which subsequently leads to the formation of autoantibodies that can be a consequence of persistent infection (Rosen & Hugo 2011). Several autoantibodies with different specificities have been identified in patients with chronic hepatitis (Metwally et al. 2012). Studies have demonstrated that chronic HCV infection may lead to a loss of tolerance to self-antigens and the consequent production of autoantibodies. HLA (chromosome 6p21) plays an important role in the regulation of the immune system and is strongly linked to hepatitis (Béland et al. 2009).

In the current study, the mean age of the patients was 43.47 years, which is in agreement with Aslam et al. (2016) and Hamid et al. (1995). In the current study, the frequency of females was higher (52.4%) as compared to males (47.6%), which is in agreement with Kumar et al. (2017) but not in agreement with Falleti et al. (2008) who found a higher percentage of males (71.8%) as compared to females (28.2%). In the current study, there was a higher mean ± SD of ALT, AST, ALP, and bilirubin in patients who were not receiving anti-HCV therapy as compared to those who were receiving anti-HCV therapy; however, there was no association between biochemical parameters and anti-HCV treatment. Nuclear antibodies that recognize and bind to DNA, i.e., anti-dsDNA antibodies, are present in autoimmune diseases like systemic lupus erythematosus (SLE) (Pisetsky 2016). In the current study, the percentage of dsDNA antibodies was 4.6%.

A study performed on a Chinese population included 393 CHC patients who also reported high ALT and AST (Ma et al. 2015). Tomer et al. (2016) suggested higher levels of AST and ALP in HCV patients who were not responding to anti-HCV therapy as compared to those who responded to therapy. However, HCV is not the sole risk factor for increased ALT and AST levels, as they are also documented in many other hepatic inflammatory conditions, e.g., non-viral hepatitis (Tarao et al. 2002; Tomer et al. 2016). The current study elucidated that ASMA, AMA, ANA, LKM-1, and dsDNA autoantibodies were not associated with gender, treatment, or duration of infection. The percentage of ASMA (17.4%) reported in the current study is in agreement with Muratori et al. (2005), but it is not in agreement with Kirdar et al. (2016) and Stroffoloni et al. (2004). The low frequency of anti-LKM-1 antibodies in the current study is similar to Clifford et al. (1995) and Kirdar et al. (2016). The percentages of ANA and AMA in the current study are not in agreement with Kirdar et al. (2016), who detected a low percentage of ANA (6.4%) and AMA (2.7%) (Clifford et al. 1995; Kirdar et al. 2016; Muratori et al. 2005; Stroffolini et al. 2004).

In the current study, the HLA- DRβ1 (*04) allele was detected in 3 (3.5%) CHC patients and there was no association between this allele and detected autoantibodies. These results are not in agreement with Umemura et al. (2014), who reported an association of DRB1*04:05 and DQB1*04:01 haplotypes with AIH susceptibility and the correlation of these alleles with elevated serum levels of IgG and ASMA. Kaur et al. (2014) also reported an association of HLA DRB1*04 and DRB1*08 with AIH type 1 in a south Indian population.

A study conducted by Czaja and Carpenter (2006) is not in accordance with the current study. They reported autoantibodies in 43% of HCV patients. Among these patients, 30% had ANA, 8% had ASMA, and 5% had both ASMA and ANA. They reported a higher frequency of ASMA in patients with HLA DR3 as compared to normal subjects. These differences could be attributed to different factors such as different genetic backgrounds, which influence the distribution of HLA alleles in CHC, while sample size and study design could be other reasons for this discrepancy (Baharlou et al. 2016).

CONCLUSION

ANA were detected in 41%, ASMA in 17.4%, AMA in 7%, LKM-1 in 5.8% and dsDNA in 4.6% of CHC patients. HLA-DRβ1 (*04) was present in 3.5% of patients; it was not significantly associated with these autoantibodies.

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