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Chronic Liver Disease and Cirrhosis Among Patients With Hepatitis B Virus Infection in Northern Portugal With Reference to the Viral Genotypes

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The prevalence of infection with hepatitis B virus in Portugal is around 1% of the population; 20–30% of those infected typically develop cirrhosis. The study focuses on the epidemiological profile of patients with hepatitis B infection and liver damage, in particular, cirrhosis. Of the 358 individuals that comprised the study, a liver biopsy was performed in 249 to identify the presence of cirrhosis. Cirrhosis was observed in 59 patients (23.7%) The Child–Pugh classification was used to assess the prognosis of cirrhosis: 3 out of the 59 patients were classified as Child–Pugh grade C, the most severe, 17 (28.8%) as grade B, and 39 (66.2%) as grade A. Patients classified as grade B were older, drank more, and showed higher levels of AST and alkaline phosphatase when compared with individuals classified as grade A. Genotypes A and D were predominant, and no significant differences with respect to genotype distribution were observed. Analysis of the hematological parameters showed that patients classified as Child's grade B had lower levels of platelets and higher levels of prothrombin time than those classified as Child's grade A. The profile of the patients with cirrhosis, including an extended number of individual characteristics, provides useful information, however, only a prospective study could evaluate definitively if liver disease is influenced by these factors. Future studies would benefit from the analysis of the impact of genotypes on liver disease, particularly genotypes A and D, the most predominant genotypes in northern Portugal. ***J. Med. Virol. 83:71–77, 2011.***

KEY WORDS: hepatitis B; genotypes; liver disease; cirrhosis; Portugal

INTRODUCTION

Cirrhosis occurs in 20–30% of those patients who are infected chronically with the hepatitis B virus (HBV). About 25% of these may develop hepatocellular carcinoma (HCC). In patients with chronic HBV and HCC, the genetic material of HBV is frequently found to be part of the genetic material of the cancer cells. The HBV genetic material enters the normal liver cells and disrupts their function, and may lead to transformation into cancerous cells. Because of the genetic mutation caused by HBV, a patient with HBV infection may develop HCC with or without cirrhosis.

Persistent hepatitis B infection affects 350–400 million people worldwide [Lee, 1997; Yuen et al., 2009]. These patients are at least 100 times more likely to develop HCC compared with uninfected individuals [Beasley et al., 1981]. HCC is the leading cause of death in patients with cirrhosis [Varela et al., 2010]. Several potential factors have been linked with a higher risk of developing HCC, including male gender, advanced age, virological factors such as hepatitis B e antigen (HBeAg) positivity and high serum HBV DNA levels [Sherman, 2005].

The association between HBV genotypes and severity of liver disease remains debatable, although one study

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has found more fibrosis in patients with genotype A than with genotype D [Halfon et al., 2006].

Portugal is populated by approximately 10 million inhabitants, with an immigrant community of around 4%. Immigrants are concentrated in the South with an epicentre in Lisbon while the North of the country has relatively fewer immigrants [MAI, 2008]. At present, there is no information on the prevalence of HBV infection in Portugal. According to the most representative research in this field, patients chronically infected with HBV comprise approximately 0.36–1% of the Portuguese population [Lecour et al., 1984; Santos et al., 2000; Ministério da Saúde, 2004].

The results of the study are part of a project in progress at a Portuguese university to characterize the epidemiological profile of the HBV in northern Portugal [Mota et al., 2009, 2010]. This study, within the ambit of a larger project, focuses on the epidemiological profile of liver damage, particularly cirrhosis, in patients infected chronically with HBV.

MATERIALS AND METHODS

Study Design

Data were obtained as part of an epidemiological study of the HBV in northern Portugal [Mota et al., 2009]. From a group of 358 individuals with chronic HBV infection, serum samples were tested for HBsAg, antibodies to HBsAg (anti-HBs), antibodies to the HBV core antigen (anti-HBc), HBeAg, and antibodies to HBeAg (anti-HBe). HBV genotypes were determined along with other biochemical and hematological parameters. The present study includes the 249 patients who underwent histological evaluation.

Patients

Patients with hepatitis B infection were identified by the presence of HBsAg in their serum for at least 6 months. The majority of patients from the North of Portugal, are treated in three hospitals in the city of Oporto: Hospital de Santo António, Hospital Joaquim Urbano and Hospital de São João. The patients in this study were examined in 2008 and 2009 in the Hospital de Santo António and Hospital Joaquim Urbano. The study was approved by the Health Ethics Committee of Hospital de Santo António, and written consent was obtained from all participants.

Clinical Findings

Information related to the demographics, clinical, histological, and medical history of the patients was retrieved from medical files.

Biochemical Tests of Liver Function and Other Blood Parameters

The blood tests considered when assessing liver function included: alanine aminotransferase (ALT, reference values 10–36 U/L at 37°C) and aspartate aminotransferase (AST, reference values 10–30 U/L at 37°C), which are contained within liver cells, gamma-glutamyltranspeptidase (GGT, reference values 10–66 U/L at 37°C), alkaline phosphatase (AP, reference values 45–122 U/L at 37°C), and alpha-fetoprotein (AFP, reference values <10.9 ng/ml). Hematological parameters like mean corpuscular volume (MCV), and platelets were also evaluated using an Advia 120 System (Bayer, Tarrytown, New York). Prothrombin time (PT) measurement was performed using an ACL TOP system (Instrumentation Laboratory Company, Lexington, MA).

Detection of Virological Markers in Serum

HBsAg, anti-HBs and anti-HBc, HBeAg, and anti-HBe antibodies were evaluated using a Vitros ECI assays (Ortho-Clinical Diagnostics, Amersham, Buckinghamshire, UK).

HBV DNA Quantitation

HBV DNA levels were determined using the Versant® HBV DNA 3.0 assay (bDNA; Bayer). The assay quantitation range is 357–17,900,000 HBV DNA IU/ml.

HBV Genotyping

HBV genotypes were identified by the Trugene® HBV genotyping kit in conjunction with the Open Gene ® DNA Sequencing System (Bayer).

Cirrhosis

Cirrhosis was evaluated by liver biopsy.

Child–Pugh Classification

This classification was used to assess the prognosis of cirrhosis. Child's grading of severity in chronic liver disease was classified along with Pugh's [1973] modifications and based upon information recorded from medical files. The classification has three grades, from A to C with grade C being the severest. The respective overall 5-year mortality rates for grades C–A are 88%, 38%, and 29%.

Statistical Analysis

Statistical analysis was performed with SPSS (version 16.0) and two-sided significance tests at the 5% level were used throughout.

Qualitative variables were described as percentages and quantitative variables as means, or geometric means where necessary. The distribution of viral load was logarithmically transformed and the results are given as geometric means with 95% confidence intervals. For the other quantitative variables, results were given as arithmetic means (±standard deviation).

Proportions were compared using the Pearson's chi-squared test (with continuity correction) as appropriate, or by Fisher's exact test. Continuous variables

were compared using the *t*-test, after log-transformation so that assumptions were met, if necessary. Multivariable analysis was not considered due to the reduced number of individuals when subdivided by cirrhosis, gender, and the Child's grading of disease severity for chronic liver disease.

RESULTS

Liver biopsy was performed on 249 patients for histological assessment. Cirrhosis was found in 59 patients (23.7%).

Genders were analyzed separately, and patients with and without cirrhosis were compared. Genotypes A and D were predominant, and no significant differences between the study groups were observed with respect to genotype distribution, in both genders (Table I). The distribution of the presumed sources of transmission of viral infection was similar among female patients, while sexual transmission was an increased factor in male patients with cirrhosis ($P = 0.013$). Alcohol intake levels (less or more than 20 g/day) were similar between the two groups with or without cirrhosis. On average, male patients with cirrhosis were older than patients without cirrhosis ($P = 0.001$), while the same was true with female patients the relationship was not significant.

In patients with cirrhosis, liver disease severity was evaluated using Child's grading of disease severity with Pugh's modifications (Table II).

Evaluation of the 59 patients, 32.2% female, was carried out according to the Child–Pugh classification. In the female group, Child's grade A classification was the most frequent (78.9%) and only one woman was classified as Childs grade C. On the other hand, an increased proportion of Child's grade B classification was observed in men (35% vs. 15.8% in women) while two were classified as Child's grade C.

Child's Grade Equivalent C

Only three patients scored sufficiently to receive a Child–Pugh classification level C, two of them being male. All patients were genotype D, and aged between 66- and 80-year old. The presumed source of infection is unknown in two patients while the third patient had a reported intrafamilial transmission of HBV infection. All patients (n = 3) were HBeAg negative, and all of them also presented viral load of HBV >357-HBV DNA IU/ml.

TABLE I. Characteristics of Patients With and Without Cirrhosis

	Without cirrhosis (n = 190)		With cirrhosis (n = 59)		
	n[a]	%	n[a]	%	*P*-value
Female					
Genotype					0.554[c]
A	20/83	24.1	7/19	36.8	
D	55/83	66.3	12/19	63.2	
E	4/83	4.8	0/19	0.0	
F	4/83	4.8	0/19	0.0	
D and F	0/83	0.0	0/19	0.0	
Presumed source of HBV infection					[d]
Perinatal	20/83	24.1	4/19	21.1	
Sexual	13/83	15.7	2/19	10.5	
Intrafamilial	29/83	34.9	6/19	31.6	
Others[b]	21/83	25.3	7/19	36.8	
Alcohol intake >20 g/day	25/70	35.7	6/18	33.3	0.850
Mean age (±SD)	44.1	±12.9	48.5	±19.1	0.354
Male					
Genotype					0.742[c]
A	37/107	34.6	13/40	32.5	
D	60/107	56.1	26/40	65.0	
E	6/107	2.8	0/40	0.0	
F	3/107	2.8	1/40	2.5	
D and F	1/107	0.9	0/0	0.0	
Presumed source of HBV infection					0.013
Perinatal	29/107	27.1	3/40	7.5	
Sexual	13/107	12.1	12/40	30.0	
Intrafamilial	23/107	21.5	10/40	25.0	
Others[b]	42/107	39.3	15/40	37.5	
Alcohol intake >20 g/day	53/96	55.2	21/37	61.8	0.872
Mean age (±SD)[c]	41.5	±13.9	50.8	±15.9	0.001

[a]Total for each variable may not sum to the sample size due to missing data.
[b]Other sources, including at least one incidence of intravenous drug use, blood transfusion, occupational exposure, iatrogenic exposure (e.g., endoscopy or coloscopy, history of surgery, acupuncture, or hemodialysis), or unknown.
[c]Evaluated only for genotypes A and D.
[d]The necessary statistical assumptions were not met.

TABLE II. Patients With Cirrhosis (n = 59) Classified by Child's Grade

Child's grade	n	%
Female		
A	15/19	78.9
B	3/19	15.8
C	1/19	5.3
Male		
A	24/40	60.0
B	14/40	35.0
C	2/40	5.0

Biochemical tests revealed ALT was elevated in two individuals, AST in all three, while only one occurrence of elevated AP, GGT, and AFP was found.

Child's Grade Equivalent A and B

Individuals classified as Child's grade A (n = 39) and B (n = 17) were compared by gender, genotype, the presumed source of infection, alcohol intake, place of birth, age, viral markers, viral load, and biochemistry parameters (Table III).

When patients with cirrhosis, were compared by gender, genotype, and the presumed source of infection, no significant differences between Child's grades A and B were found. Individuals classified as Child's grade B were older and had a significantly higher proportion of patients with alcohol intake above 20 g/day ($P \leq 0.004$). Patients classified as Child's grade B showed a tendency to have higher proportions of elevated biochemistry parameters like AST, AP, GGT, and AFP when compared with individuals classified as grade A.

Hematological parameters were also compared (Table III). Patients classified as Child's grade B had significantly lower levels of platelets ($P < 0.001$) and significant higher levels of PT ($P = 0.013$) than patients classified as Child's grade equivalent A.

DISCUSSION

This study is one of the first attempts to describe the epidemiological profile of liver damage, particularly cirrhosis, in patients infected chronically with HBV in Portugal. Both patients with and without cirrhosis formed part of the study.

All patients in the study were from the North of Portugal. In the sample of patients infected with HBV

TABLE III. Comparison of the Characteristics of Patients With Cirrhosis Classified as Child's Grade A and Child's Grade B (n = 56)

	Child–Pugh A (n = 39)		Child–Pugh B (n = 17)		
Characteristics	n[a]	%	n[a]	%	*P*-value
Gender					0.222
Female	15/39	38.5	3/17	17.6	
Male	24/39	61.5	14/17	82.4	
Genotypes					
A	15/39	38.5	5/17	29.4	0.844[c]
D	24/39	61.5	11/17	64.7	
F	39	0.0	1/17	5.9	
Presumed source of HBV infection					
Perinatal	7/39	17.9	17	0.0	[c]
Sexual	10/39	25.6	4/17	23.5	
Intrafamilial	10/39	25.6	5/17	29.4	
Others[b]	12/39	30.8	8/17	47.1	
Alcohol intake >20 g/day	12/36	33.3	13/16	81.2	0.004
Mean age (±SD)	44.5	±16.6	59.9	±11.4	0.001
Viral markers					
Anti-HBc positive	39/39	100.0	17/17	100.0	[d]
HBeAg negative	24/39	61.5	14/17	82.4	0.222
Anti-HBe positive	22/39	56.4	14/17	(82.4)	0.119
Viral load geom. mean (95% CI)	1,400,024.1	450,584.1–4,350,059.2	384,269.7	60,657.6–2,434,373.5	0.212
Biochemistry parameters greater than reference values					
ALT	27/39	69.2	10/17	58.8	0.653
AST	23/39	59.0	15/17	88.2	0.065
AP	3/37	8.1	6/17	35.3	0.021
GGT	11/37	29.7	9/17	52.9	0.181
AFP	2/32	6.2	3/16	18.8	0.316
Hematological parameters mean (±SD)					
Platelet × 10^9/L	163.3	±57.8	100.1	±48.2	<0.001
MCV	90.5	±8.8	94.0	±6.5	0.164
PT	13.2	±3.0	16.1	±5.5	0.013

[a]Total for each variable may not sum to the sample size due to missing data.
[b]Other sources, including at least one incidence of intravenous drug use, blood transfusion, occupational exposure, iatrogenic exposure (e.g., endoscopy or coloscopy, history of surgery, acupuncture or hemodialysis), or unknown.
[c]Evaluated only with genotypes A and D.
[d]The necessary statistical assumptions were not met.

who underwent histological evaluation (n = 249) the results showed that there was a considerable proportion with an increased incidence of liver damage, while 23.7% of the patients had cirrhosis. Genotypes A and D were predominant in patients with or without cirrhosis. A previous study in the North of Portugal [Mota et al., 2009] found genotypes A and D to be predominant, with genotype D accounting for 60.1% and genotype A accounting for 30.1% of the patients evaluated. In Spain, the only country that borders Portugal, genotypes A and D have been also found to be prevalent [Echevarria and Leon, 2004; Basaras et al., 2007], although the relationship with the stage of cirrhosis is undocumented. A study in India found that genotype D was associated with more severe liver diseases when compared with genotype A. Genotype D was more prevalent in HCC patients less than 40 years of age, when compared with asymptomatic carriers [Thakur et al., 2002]. Similar results are likely to be found in the North of Portugal, given a suitably large sample size.

In this study, a statistical association was not found between cirrhosis and genotypes of HBV ($P \leq 0.554$), or alcohol consumption level ($P \leq 0.850$), for both male and female patients. Comparison between patients without and with cirrhosis showed a tendency for patients with cirrhosis to be older. Recent studies have shown that, older age, male gender, and cirrhosis were the major risk factors associated with the development of HCC [Lok, 2009].

In this study a classification system developed to assess the severity of liver cirrhosis, the Child–Pugh grade, was chosen to evaluate patients. In the study sample, 3 out of the 59 patients were classified as Child–Pugh grade C, the most severe, 17 (28.8%) as grade B and 39 (66.2%) as grade A.

To understand better what factors are associated with the severity of liver disease those patients with cirrhosis classified as Child–Pugh grade B were compared with the group classified with the less severe grade A, in terms of HBV genotypes, patient demographics and alcohol intake, transmission routes, virological markers for HBV, viral load, liver enzymes, and hematological parameters. In this study, a statistical association was not found between severity of liver disease and gender ($P = 0.222$), genotypes, ($P = 0.844$), eventual transmission routes of infection, HBeAg negativity ($P = 0.222$), anti-HBe positive ($P = 0.119$), and viral load ($P = 0.212$). All patients were anti-HBc positive. The analysis showed that patients classified as Child–Pugh grade B were older ($P = 0.001$) and had a significantly higher percentage of alcohol intake above 20 g/day ($P = 0.004$) when compared to individuals classified as Child–Pugh grade A. The same trend has been observed previously when studying the relationship between the severity of liver disease and age and alcohol consumption [Mota et al., 2010]. While this previous study reported a relationship between alcohol consumption and liver disease severity, a relaxed definition of liver disease was used, including patients with and without histological evaluation, and patients without disease reports. The present study, restricted only to patients with cirrhosis, achieved the same positive association between age, alcohol intake and severity of liver disease. Age and alcohol consumption were also reported as risk factors associated with HCC in many studies from different countries, including Portugal [Pereira et al., 1994; Oliveira et al., 2001; Wang et al., 2003; Chung et al., 2007].

Hepatitis B infection may influence the development of liver disease in heavy alcohol drinkers in several ways. Firstly, persistent infection with HBV might encourage alcohol-induced liver damage, and evidence exists that this may occur at fairly low levels of alcohol intake [Villa et al., 1982]. On the other hand, chronic active hepatitis associated with HBsAg may coexist with alcoholic liver disease, and the two disease processes would probably result in more rapid progression to cirrhosis. For this reason, a vaccination program would be of great benefit to excessive drinkers, as infection with HBV and hepatitis C virus (HCV) are major contributing factors towards the development of HCC [Nalpas et al., 1998]. Alcohol-dependent patients under treatment or attended to by emergency hospital services, are opportunities for preventive intervention. Such patients should be checked for viral diseases like hepatitis C, human immunodeficiency virus (HIV), and HBV infection. In all individuals without evidence of infection with HBV, a vaccination should be implemented.

The pattern observed for patients without HBeAg and viral load, in patients with chronic hepatitis B, seems to be predominant in Mediterranean countries. Additionally, some investigators have found that HBV infection remained active after seroconversion to anti-HBe in nearly 40% of patients [Rodriguez-Frias et al., 1995; Grandjacques et al., 2000]. In France, an increasing prevalence of HBeAg negative was observed [Zarski et al., 2006], in accordance with this study of Portugal, with probable basal promoter core or a precore region mutations (two individuals presented anti-HBc negative). The knowledge of this characteristic is very important for optimizing clinical management and future therapeutic trials for chronic hepatitis B. Particularly, with respect to HBV replication status, HBV genotype and mutations in the basal core promoter region play an important role in the HCC development [Lok, 2009]. Many factors seem to contribute to the development of HCC: host and viral risk factors associated with increased risk of cirrhosis or HCC include older age (longer duration of infection), alcohol consumption, smoking, HBV genotype C, high levels of HBV DNA and concurrent infection with hepatitis-like HCV, hepatitis D virus (HDV), or HIV [Fattovich et al., 1995; McMahon et al., 2001; Fattovich, 2003; Yim and Lok, 2006].

This Portuguese study showed that ALT levels in patients with Child's grade A and B had similar values. On the other hand, Child's grade B patients, showed a higher proportion with elevated levels of AST and AP, when compared with individuals classified as grade A.

Even in this Portuguese study, patients classified as Child's grade equivalent B had significantly lower levels of platelets ($P < 0.001$) and significant higher levels of PT ($P = 0.013$) compared to the Child's grade equivalent A. Thrombocytopenia is considered as a valid surrogate of cirrhosis and a valid marker for the identification of individuals at high-risk for HCC [Lu et al., 2006]. Although not significant, Child's grade B individuals presented elevated values for GGT and AFP.

In conclusion, the present study examined the associations between cirrhosis, and an extended number of patient's characteristics, providing useful information on the profile of patients infected with HBV in northern Portugal. The group of patients with cirrhosis, classified as Child–Pugh grade B, was older and showed elevated levels of alcohol intake. Comparison of patients classified as Child–Pugh grade B with individuals classified as Child–Pugh grade A, showed a higher proportion of biochemical parameters particularly, AST, AP, GGT, and elevated AFP, with hematological parameters showing lower platelet levels and higher PT values.

In the evaluation of cirrhosis, liver biopsy continues to be the preferred diagnostic method, but the appearance of "stiffness" measurement techniques will probably be the eventual future diagnosis of cirrhosis. However, additional studies are needed for the evaluation of liver fibrosis possibly based on liver "stiffness" measurements, and cutoff values must be defined for categorizing the different stages of cirrhosis.

Although no association between HBV genotypes and severity of liver disease was observed with the sample evaluated, it would be important in future investigations to determine the impact of genotypes on liver disease, particularly cirrhosis.

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