Original Article

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Study of Histomorphological Characteristics and it's Correlation with Clinical, Biochemical, Serological and Immunohistochemical Parameters in Incidentally Detected Hepatitis B Patients

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ABSTRACT

Background: India lies in intermediate endemicity zone for hepatitis B virus (HBV) infection and constitutes the second largest global pool of HBV infection worldwide. Hepatitis B has a varied clinical presentation ranging from clinically asymptomatic state to cirrhosis and hepatocellular carcinoma. A significant liver injury can occur, without accompanying elevation in alanine transaminase (ALT) and HBV DNA levels, especially in incidentally detected asymptomatic hepatitis B subjects (IDAHS). Hence, a role of liver biopsy to be incorporated with other investigations is debatable, but important to initiate antiviral therapy.

We explored correlation between histomorphological outcomes with various clinical, biochemical, serological and immunohistochemical parameters in IDAHS.

Methods and Material: Total 113 patients were consecutively selected over a period of 4.5 years. Serological work-up for HBsAg, Anti-HBeAg, Anti-HBeAb, Anti-HBcAb, and HBV DNA levels were done as per resources. A liver biopsy was done in each patient after a written consent. Ishak's scoring system was used to assess histological parameters. Immunohistochemistry (IHC) was done for HBsAg and HBcAg. Appropriate statistical tests were applied.

Results: The mean age of the patients was 30 years with a male to female ratio of 3:1. A higher necro-inflammatory activity (NIA >3) correlated with high ALT (>40 U/l), HBV DNA (>105 copies/ml) and fibrosis (F ≥2). HBeAg-positive patients had significantly higher NIA and HBV DNA levels. Anti-HBeAb delineated association with ALT (≤40 U/l) and low HBV DNA but more severe fibrosis (F≥2). Steatotic changes were noted in 52.2% biopsies. IHC for HBsAg and HBeAg showed positivity in 82.7% and 39.2% of cases respectively with a significant correlation between membranous pattern of HBsAg staining and serum HBV DNA levels.

Conclusion: IDAHS represent tip of the iceberg of major HBV infection reservoir. A liver biopsy is a useful additional tool with other parameters to further tailor the therapy in such asymptomatic patients.

Keywords: *Hepatitis B Virus, Hepatitis B Surface Antigen, Hepatitis B e Antigen, Viral DNA, Alanine Transaminase.*

Introduction

Worldwide prevalence of hepatitis B virus (HBV) varies significantly among different population. In India, prevalence of hepatitis B surface antigen (HBsAg) in general population ranges from 2% to 7%, which lies in intermediate endemicity zone.[1] Of the estimated 360 million worldwide chronic carrier, India accounts for about 50 million, forming the second largest global pool of chronic HBV infections.[2] In India transmission is mostly acquired through childhood horizontal spread due to sub-optimal hygiene and crowded living conditions and perinatal transmission of infection from mother to infants is not an important route.[1] Hepatitis B disease has a varied clinical presentation ranging from clinically asymptomatic state to the development of cirrhosis and hepatocellular carcinoma depending upon the phase. Patient may present (a) in a state of immune tolerance, (b) HBeAg-positive chronic HBV, (c) HBeAg-negative chronic HBV, or (d) as an inactive HBsAg carrier.[3, 4] Chronic HBV (both HBeAg-positive and HBeAg-negative) patients have a relatively higher chance of developing various complications.[5]

All major liver organizations, such as the American association for the study of liver diseases (AASLD), the European association for the study of the liver (EASL), and the Asian-Pacific association for the study of the liver (APASL), consistently recommend therapy for patients with liver damage and complications.[6, 7] However, these recommendations for treatment of chronic hepatitis B

patients are not always appropriate to apply in most of the developing countries.[8]

Although incidentally-detected asymptomatic HBsAg-positive subjects (IDAHS) appear healthy at the time of presentation, varying proportions have evidence of liver disease on biopsy.[9] Though ALT, HBV DNA levels and HBeAg status determination serve as important means to predict the extent of disease in these patients, nevertheless a liver biopsy plays an important role to ascertain amount of injury and also decision making to plan antiviral treatment.[10-12]

The limited number of liver biopsies in IDAHS patients, more so in developing countries and the scarce literature on correlation between various histological indices with serological and viral markers provided us insight for this study.

We started this study with an aim to correlate various measurable histological events with serological and viral parameters in IDAHS patients to guide clinicians in deciding appropriate antiviral therapy.

Methods and Materials

This paper is approved by departmental review board. Total 137 consecutive patients presented in the liver clinic of Gatroenterology outpatient department of the institute over a period of 4.5 years from January, 2010 to June, 2014 were included in the study. All patients were apparently healthy without any features of liver-related diseases during their first visit to the hospital. These patients came to the hospital for a routine check-up before foreign trip (as asked by embassy officials for visa), during pregnancy, after household contacts, health personnel after accidental exposure, referred from blood bank etc. All the patients who found to be HBsAg positive were asked to follow different public health measures. Also, patients were requested to seek HBsAg testing after 6 months and to visit in case of any illness. When HBsAg was detected 6 months after the first test, the patients were regarded as being chronically infected with HBV, and enrolled.

Patients with the past history of chronic liver disease or decompensation of liver function, alcohol consumption more than 20 g/day, hepatotoxic drug, any systemic illnesses such as diabetes mellitus, and those having co-infection with HIV or hepatitis C virus were excluded from the study. Physical parameters such as body mass index and waist hip ratio was measured in each patient at the time of registration. Ultrasonography report, if available was also included.

Biochemical and Serological Tests: Routine haemogram, lipid profile, serum insulin, fasting blood sugar and liver function tests were done on all patients. The cutoff for the upper limit of normal (ULN) was ALT > 40 U/l. HBsAg was assessed using a commercial ELISA kit (Diasorin, Fallugia, Italy). HBeAg and anti-HBe antibody were tested using an ELISA kit (Abbott Labs, Chicago, Ill., USA).

Serum HBV DNA was quantified using an RT-PCR kit (Amplicon HBV Monitor Assay, Roche Molecular Systems, Calif.,USA). The lower limit of detection was 250 copies of HBV DNA/ml. A level of $>10^5$ copies/ml was considered as active/replicative infection.

Liver biopsy: A prior written informed consent was taken from all the patients before liver biopsy. Percutaneous liver biopsies from all the patients were obtained under local anesthesia using 16G Tru-Cut needles (Cardinal Health, McGaw Park, Ill, USA). The liver biopsies at least 1 cm long with ≥6 portal triads were included. The liver biopsies were fixed in 10% buffered formalin and four micron sections were stained with hematoxylin and eosin (H&E), masson's trichrome and orcein stains. Necro-inflammatory activity (NIA) and fibrosis stage were assessed using the Ishak's scoring system. Extent of hepatitis was graded as mild (NIA≤3), and significant (NIA≥4), while fibrosis was staged as follows: F0, no fibrosis; F1, mild portal fibrosis with/without septa; F2, marked portal fibrosis with/without septa; F3, portal fibrosis with occasional P-P bridging septa; F4, portal fibrosis with numerous bridging septa; F5, marked bridging fibrosis with occasional nodules; F6, cirrhosis. Immunohistochemistry (IHC) was performed on 4μ thick formalin fixed, paraffin-embedded sections using antibodies directed against HBsAg (Neomarker) and HBcAg (Neomarker). Antigen retrieval for HBsAg was done in a microwave oven using citrate buffer at pH 6.0 and streptavidin biotin conjugate immunoperoxidase method was used. For each batch, appropriate positive controls were taken and for negative controls primary antibodies were omitted.

Statistical Analyses: Data were analyzed using SPSS version 17 software package (SPSS, Inc., Chicago, IL). Statistical analyses were performed using Chi square and Fisher exact tests for categorical variables. Student *t* test or 1-way analysis of variance was used for group comparisons of parametric quantitative data. Differences were considered significant at $p< 0.05$.

Results

Baseline characteristics of patients (Table 1). Of the 137 patients, 113 were found eligible after putting exclusion criteria's. The mean age of the patient was 30 years; 84 (74.3%) were male and the other 29 (25.7%) were female. HBeAg status did not revealed significant correlation with age. The median level of serum ALT was 47 U/l (range 11–

790 U/l). ALT was higher than the cut off for the ULN in 65.6% subjects. Of the 113 patients, liver biopsies showed variable amount of portal inflammation in all, ground glass hepatocytic changes in 74 (65.4%), and lobular inflammation in 92 (81.4%) (Table 2). Both ground glass changes with concurrent lobular inflammation were noted in 57 (50.4%) patients. Interface activity, focal lymphoid aggregate and ductal injury was noted in 8 (7%), 13 (11.5%) and 7 (6.1%) biopsies respectively. Abdominal ultra-sonography reports were available in 24 patients, 5 (20.8%) of which showed abnormal echotexture. High NIA delineated significant correlation with high ALT levels, HBV DNA and fibrosis (Table 3). HBeAg status was known in 90 patients, of which 43 (47.7%) were HBeAg-positive.

Anti-HBe antibody (Anti-HBeAb) levels were investigated in 58 patients, 30 (51.7%) of which showed positivity (Table 4). Anti-HBeAb positivity was more common in patients with low ALT (≤40 U/l) and HBV DNA levels ($<10^5$ copies/ml) but more severe fibrosis (F≥2).

Anti HBc antibody (Anti-HbCAb) was measured only in 17 patients, 12 (70.5%) of which were positive. Comparatively higher HBV DNA levels were noted in HBeAg-positive against HBeAg-negative patients (71.4 vs. 28.6%, $p < 0.0001$) (Table 5).

Similarly, a higher NIA was seen among HBeAg-positive patients. Also, in HBeAg-positive group, 21 (48.8%) patients had both higher serum ALT and HBV DNA levels, in comparison to HBeAg-negative group, 6 (12.8%) and the difference was statistically significant (p value <0.0001).

Steatotic changes were noted in 59/113 (52.2%) patients, which varied considerably ranging from 5% to 85% of the hepatocytes with a meadian of 10%. Steatosis amount and pattern did not showed differences with HBeAg status. IHC for HBsAg and HBcAg showed positivity in 67/81(82.7%) and 33/84 (39.2%) of the biopsies respectively (Figure 1). Both the antibodies showed three pattern of the staining including cluster, diffuse and scattered. In addition to cytoplasmic positivity, HBsAg also showed membranous staining in 24/67 (35.8%) of the biopsies. HBcAg revealed both nuclear and cytoplasmic positivity in 10/33 (39.2%) of the cases. The membranous pattern of HBsAg showed a positive correlation with the serum levels of HBV DNA (p=0.018).

Table 1: Baseline characteristics of the hepatitis B patients

Parameters		Values
No of patients		113
Age, years		29.9±11.9
Male		74.3%
Female		25.7%
HBeAg	positive	47.8%
	negative	52.2%
Anti-HBeAb	positive	51.7%
	negative	48.3%
Anti-HBcAb	positive	70.6%
	negative	29.4%
ALT levels	Normal	34.4%
	Raised	65.6%
HBV DNA levels	≤10^5 (copies/ml)	41.2%
	>10^5 (copies/ml)	58.8%
Ultrasound	Normal echotexture	79.2%
	Coarse echotexture	20.8%

Table 2: Histopathological characteristics, Ishak's staging and grading and immunohistochemistry (HBsAg & HBcAg) of the liver biopsies in the hepatitis B patients.

Parameters		Values
Portal inflammation		100%
Ground glass changes		65.5%
Lobular inflammation		81.4%
Interface activity		7.1%
Lymphoid aggregate		11.5%
Duct injury		6.2%
NIA score	1-3	51.9%
	>3	48.1%
Fibrosis stage	0-1	65.9%
	≥2	34.1%
Steatosis (%) (n=59)	1-9%	37.3%
	10-30%	44.0%
	>30%	18.7%
HBsAg positive (Total/C/C+M/M)		82.7%/64.1%/32.8%/2.9%
HBcAg positive (Total/N/C+N)		39.2%/69.6%/30.3%
'NIA'- necro-inflammatory activity, **'C'**- cytoplasmic, **'M'-** membranous, **'N'**- nuclear		

Table 3: Correlation of NIA with ALT levels, serum HBV DNA and fibrosis.

Parameters	NIA (≤3)	NIA (≥4)	P value
ALT (≥1ULN)	54.1%	78.4%	0.0001
HBV DNA (≥10^5 copies/ml)	44.1%	66.7%	0.04
HBeAg+	22.9%	71.9%	0.0001
Fibrosis stage (High ≥2)	10.7%	65.8%	0.0001
Anti-HBeAb+	70.0%	45.5%	0.09

Table 4: Comparison of histological, serological and virological parameters between HBeAg-positive and HBeAg-negative patients.

Parameters		HBeAg-positive	HBeAg-negative	P value
No of patients (n=90)		43	47	
ALT levels	Normal	28.9%	46.7%	0.98
	Raised	71.1%	53.3%	
HBV DNA levels	≤10^5 (copies/ml)	14.6%	66.7%	0.0001
	>10^5 (copies/ml)	85.4%	33.3%	
NIA score	1-3	32.3%	75%	0.0001
	>3	67.7%	25%	
Fibrosis stage	0-1	60.0%	73.0%	0.26
	≥2	40.0%	27.0%	
Steatosis (%) (n=46)	1-9%	74.4%	63.8%	0.17
	10-30%	23.3%	23.4%	
	>30%	2.3%	12.8%	
Anti-HBeAb	Positive	29.6%	80.0%	0.0001
	Negative	70.4%	20.0%	

Table 5: Comparison of histological, serological and virological parameters between AntiHBeAb-positive and AntiHBeAb-negative patients

Parameters		AntiHBeAb positive	AntiHBeAb negative	P value
No of patients (n=58)		30	28	
ALT levels	Normal	48.3%	18.5%	0.01
	Raised	51.7%	81.5%	
HBV DNA levels	≤10^5 (copies/ml)	63.0%	7.1%	0.0001
	>10^5 (copies/ml)	37.0%	92.9%	
NIA score	1-3	58.3%	33.3%	0.10
	>3	41.7%	66.7%	
Fibrosis stage	0-1	56.0%	89.5%	0.01
	≥2	44.0%	10.5%	
Steatosis (%) (n=29)	1-9%	46.7%	35.7%	0.72
	10-30%	46.7%	50.0%	
	>30%	6.7%	14.3%	
HBeAg	Positive	28.6%	79.2%	0.0001
	Negative	71.4%	20.8%	

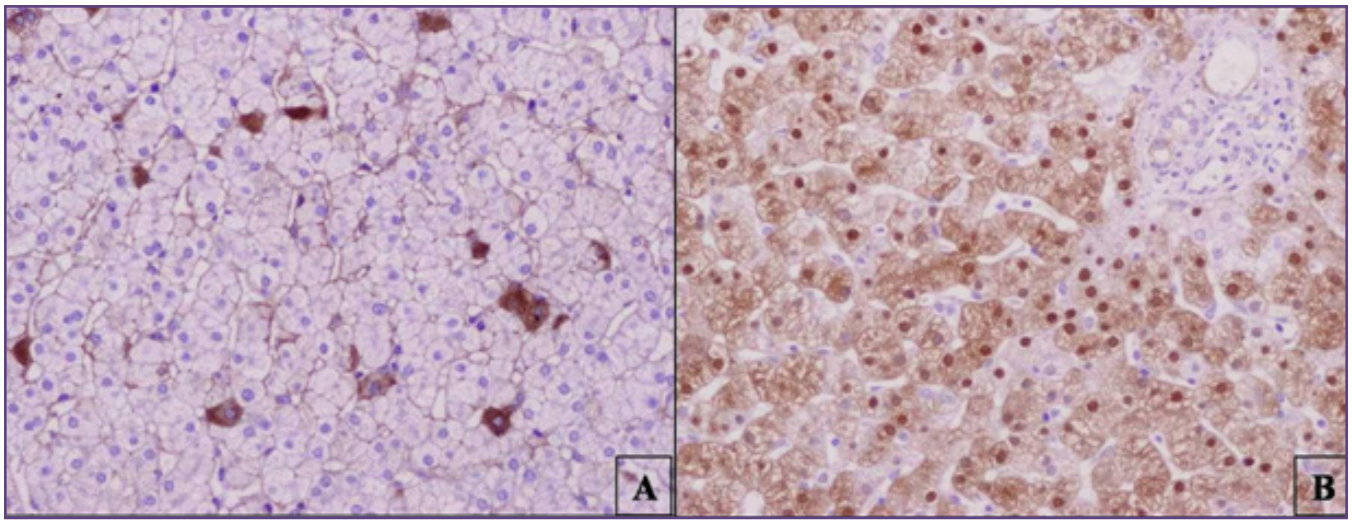

Fig. 1: Photomicrographs showing cytoplasmic and membranous immunopositivity for HBsAg (A) and nuclear & cytoplasmic positivity for HBcAg (B) (X 200 each).

Discussion

India is the second largest global pool of chronic hepatitis B (CHB) carriers. Because of socio-economic hurdles, millions of these patients are unaware of HBV infection and serve as an important source of reservoir. There are no effective screening system and treatment guidelines for these patients. Since these patients are asymptomatic, they do not seek medical attention till complications develop. We endeavor to find significance of each histological change in the liver biopsy including portal inflammation, ground glass change, lobular inflammation, lymphoid aggregate, duct injury and steatosis separately. We found ground glass change in ~65% of the biopsies which is a characteristic feature in CHB. Interestingly, in this study, we found steatosis in 52% (n=59) of the biopsies, out of which 62% had steatosis ≥10% and 11 patients among them with >30% steatosis, which is more characteristic of chronic hepatitis C (CHC). However, no significant association was found between extent of steatosis and NIA.

The higher positivity rate in liver biopsies for HBsAg (82.7%) than for HBcAg (39.2%), explain selective secretion of HBsAg and, inactivation of more immunogenic portions like core genes during the viral genome integration, thus evading immunologic attack and elimination.[13]

Mukhopadhya et al described scattered, cluster and sheet patterns of staining both for HBsAg and HBcAg, which were also seen in this study.[14] Earlier, membranous staining of HBsAg has been reported to be associated with active viral replication and disease activity.[15] We found a significant positive correlation of membranous HBsAg staining with higher serum levels of HBV DNA (p=0.018).

In our study, 21 (18.5%) patient had high ALT (above ULN), along with HBV DNA (>10^5) and NIA (≥4) which is higher than 8.5% described in study by Mahtab et al.[12]

This is the first study which explored the role of other immunological factors such as Anti-HBeAb and Anti-HBcAb in the pathogenesis of CHB. Anti-HBeAb showed a positive correlation with normal ALT and low HBV DNA levels (<10^5 copies/ml) but with distinctly higher fibrosis stage (F ≥2) (p <0.016).

Viral load, HBeAg status, ALT levels and NIA are the important indicators of liver damage and serve to guide treatment.[6, 7] In this study, we found a good correlation between these parameters. The extent of liver injury however does not always correlate with the ALT and HBV DNA levels.[16] Also, since there is a complex interplay between HBV, hepatocytes and host immune system, these markers fluctuate markedly in the course of disease.[12] ALT levels are also influenced by body mass index (BMI), metabolism and time of measurement.[17, 18] Earlier, studies supported that even in immunotolerant phase and with relatively normal ALT levels significant liver fibrosis and necro-inflammatory changes can occur.[19, 20] Recently, Kumar et al showed that IDAHS display variable amount of liver injury with normal level of biochemical (ALT), virological (HBV DNA) and immunological (HBeAg) markers.[11]

In India and other developing nations, HBV infection mostly acquired in early childhood, reflecting a longer period of insult to hepatocytes in comparison to developed countries, where most of the HBV infections trigger in adult life. Early onset of infection, itself explain more likelihood of severe liver injury than western population. So, EASL, and AASLD recommendations, based on western population with main consideration of biochemical and virological factors to commence antiviral therapy, should not be extrapolated in the developing world.

About 10% patients with HBV DNA levels <10^5 copies/ml showed significant fibrosis (F≥2). Earlier, also patients with <10^5 copies/ml have shown to carry risk for progression to cirrhosis or hepatocellular carcinoma.[21, 22] So, the cut-off of >10^5 copies/ml for HBV DNA levels may not be justified in many patients.

So, we encourage and support EASL & Lok and McMahon recommended treatment guidelines based on serum ALT levels, HBV DNA levels, and HBeAg status but a liver biopsy can be corroborated to further tailor the therapy. Rather, a personalized approach, considering regional, demographic, racial and socio-economic factors, may benefit maximum in these patients. Also, there is a need to sensitize healthcare cadre and governments towards the epidemic level of HBV infection in developing countries, and develop a global system of screening programme to curtail the dreadful further spread of this infection.

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