Evaluation of HBx Antigen in Patients with Chronic Hepatitis B Virus Infection

Amal F. Mahdi[1] *PhD*, **Aseel H. Al-Khezrachi[2]** *MSc*, **Haidar A. Shamran[3]** *PhD*, **Asmaa B. Al-Obaidi[4]** *PhD*

[1]Public Health Laboratory, Kirkuk, Iraq, [2]Baghdad-Al-Karkh Medical Center, [3]Medical Research Unit, College of Medicine, Al-Nahrain University, Baghdad, Iraq, [4]Dept. of Microbiology, College of Medicine, Al-Nahrain University, Baghdad, Iraq

Abstract

Background	Hepatitis B virus (HBV) is a serious public health problem worldwide and major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC).
Objective	To assess the association of the role hepatitis B x antigen (HBxAg) in chronic HBV; active and inactive patients, and complicated patients.
Methods	Ninety blood samples from patients who had chronic HBV were enrolled in this study. Serum samples were screened by enzyme linked immunosorbent assay (ELISA) technique for the detection of HBxAg. Viral nucleic acid was extracted from these 90 samples, and plasma HBV viral load was investigated by real time-polymerase chain reaction (RT-PCR).
Results	The overall prevalence of HBxAg was 26.7% with a significantly higher prevalence in active than inactive patients.
Conclusion	The high HBxAg prevalence rates in active chronic patients raise the possibility of increasing risk of disease progression.
Keywords	Hepatitis B virus (HBV), hepatitis B X antigen (HBxAg), hepatocellular carcinoma (HCC)
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List of abbreviations: AUC = Area under the curve, ELISA = Enzyme-linked immunosorbent assay, HBxAg = Hepatitis B X antigen, HBV = Hepatitis B virus, HCC = Hepatocellular carcinoma, ROC = Receiver operating characteristic, RT-PCR = Real time polymerase chain reaction

Introduction

Hepatitis B virus (HBV) is a small, enveloped virus with a partially double-stranded DNA genome of the family Hepadnaviridae [1]. Despite the availability of an effective prophylactic vaccine for nearly three decades, HBV remains the cause of a number of important public health problems [2].

Globally, there were an estimated 248 million persons with chronic HBV infection in 2010 and approximately 686,000 deaths were attributed to complications associated with chronic HBV infection in 2013 [3]. Chronic HBV infection remains the most important risk factor for hepatocellular carcinoma (HCC) worldwide, with the level of viremia being the number one risk factor [4].

Hepatitis B x antigen (HBxAg) is a protein of 154 amino acids, could cause enhanced colony formation or transformation of cells in vitro in various cell lines. This antigen has been shown to activate gene expression via oncogenic Ras

signaling by increasing TATA binding protein levels [5].

HBxAg has been reported to modulate the expression and activities of numerous genes, as well as epigenetic molecules (e.g., miRNAs and lncRNAs) and events (e.g., methylation and acetylation), leading to the deregulation of various pathways and function [6]. This antigen has been shown to associate with the tumor suppressor gene product p53 and inhibits its function.

The objective of this study is to assess the associated role of HBxAg in chronic HBV active and inactive patients and complicated patients.

Methods

In this case control study, blood samples were obtained from patients attending the Gastroenterology and Hepatology Center of Medical City from September 2018 to April 2019. This study was approved by the Institutional Review Board (IRB) of the College of Medicine, Al-Nahrain University, approval code number 64. The population consisted of ninety patients who had chronic HBV; divided into two groups: first uncomplicated 62 (68.9%) and second group with complicated HBV infection 28 (31.1%) (fibrosis, cirrhosis, and hepatocellular carcinoma (HCC)).

A total of 5 ml of venous blood for serum separation were withdrawn, all the 90 sample were tested for HBxAg by HBxAg enzyme linked immunosorbent assay (ELISA) Kit (My BioSource System San Diego, CA 92195-3308 USA).

Also, the viral load of all samples was quantified by first Viral Nucleic Acid Extraction Kit II (Geneaid-Tiwan), which was designed specifically for efficient purification of viral DNA and viral RNA from cell-free samples such as serum and plasma, and then Bosphore® HBV Quantification Kit (Anatolia-Turkey) have been used to detect and quantify hepatitis B virus DNA.

Detection of HBV DNA using quantitative real time polymerase chain reaction (RT-PCR)

Viral DNA extraction was conducted using Viral Nucleic Acid extraction kit (Geneaid-Tiwan) with two hundred μl viral DNA, which was extracted through three main steps lysis, nucleic acid binding, wash, and finally elute the purified nucleic acid. Hepatitis B virus was quantified by HBV Quantification kit (Bosphore®- Turkey). The kit contains positive and negative control, four external quantitation standards and PCR master mix, which contains a highly specific and accurate HotStarTaq DNA Polymerase, the PCR buffer, the dNTP Mix, the HBV-specific forward and reverse primers and a dual-labeled probe, and the internal control-specific forward and reverse primers and a dual-labeled probe.

Total volume of PCR preparation contains 15 μl of the master mix and 10 μl of DNA (sample/ standard/positive or negative control) was added into the PCR tubes or strips, then loaded into the RT-PCR machine (Magnetic Induction Cycler (MIC qPCR), BMS-Australia). The thermal cycler composed of an initial denaturation at 95 °C for 14:30 min, denaturation at 97 °C for 00:30 min, then annealing and synthesis at 54 °C for 01:30 min and hold finally at 22 °C for 05:00 min, and by the use of RT-PCR system software program calculated the baseline cycles and the threshold.

Detection of HBxAg by ELISA

Fifty μl from positive and negative control and 10 μl of testing sample were added to ELISA well then Sample Diluent 40 μl was added to testing sample well, one hundred μl of HRP-conjugate reagent was added to each well, incubated for 60 minutes at 37 °C., after that washed with washing solution, then Chromogen solution A 50 μl and chromogen solution B 50 μl were added to each well and incubated for 15 minutes at 37 °C. Finally, 50 μl Stop Solution was added and read at 450 nm using a microtiter plate reader.

Statistical analysis

The data were processed using statistical package for social sciences (SPSS) version 16.0.0, Microsoft Excel 2010, and Graphpad Prism version 7.04. Accordingly, the proper statistical tests were used. Student t-test and analysis of variance (ANOVA) test were used for parametric data to measure the significance of difference in means taking into account whether variables of analysis sharing different or equal variance. The diagnostic performance of a test or the accuracy of a test to discriminate true positive from false positive cases was evaluated using Receiver Operating Characteristic (ROC) curve analysis.

Results

The mean HBV viral load was 5.029×10^9 copies/ml, with a range between 166.8 to 3.13×10^{11}, according to Terrault *et al.* (2018) [7], chronic HBV patients were divided into active and inactive infections (median viral load was 4.9194×10^4), and in this study 41 (45.6%) patient had active, and 49 (54.4%) patients had inactive chronic hepatitis B (CHB) infections, as shown in table (1).

Table 1. Frequency of patients according to the type chronicity

Type of chronicity	Frequency	%
Active	41	45.6
Inactive	49	54.4
Total	90	100.0

Results of ELISA study of HBxAg in patients with chronic HBV infection

This study found that HBxAg was positive in 24 (26.7%) of patients with chronic HBV infection. The current study revealed a highly significant correlation between positive HBxAg and active HBV infection p<0.001, as shown in table (2).

Analysis by ROC was carried out for estimating the sensitivity and specificity of the cutoff at which chronic hepatitis patients are considered positive for HBxAg when the viral load of HBV is equal or higher than the specified cut off value. The area under the curve (AUC) for HBV was 0.768, and P <0.001. The optimal cut-off value was 5115.9 with sensitivity and specificity 82.6%, 64.2% respectively, 95% confidence interval equal to 0.665-0.871 as illustrated in table (3) and figure (1).

Table 2. Correlation of HBxAg with sex, treatment, chronicity, and complication

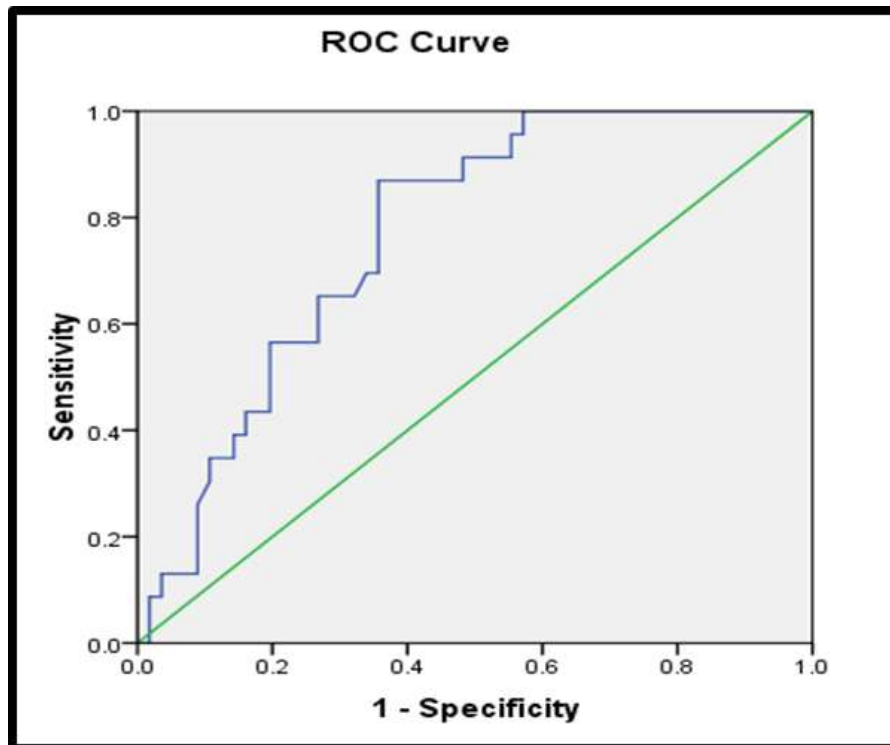| Variables		HBxAg				Total	
		-ve		+ve			
		No.	%	No.	%	No.	%
Sex	male	45	80.4	11	19.6	56	100
	female	21	61.8	13	38.2	34	100
		Chi-sq: P=0.48					
		Correlation r=+0.2, P=0.054					
Treatment	No	56	70.9	23	29.1	79	100
	Yes	10	90.9	1	9.1	11	100
		Fisher exact: P=0.15					
		Correlation r=-0.14, P=0.16					
Chronicity	inactive	46	69.7	3	12.5	49	100
	active	20	30.3	21	87.5	41	100
		Chi-sq: P<0.000					
		Correlation r=+0.5, P<0.000					
Complications	No	44	71.0	18	29.0	62	100
	Yes	22	78.6	6	21.4	28	100
		Chi-sq: P=0.45					
		Correlation r=-0.08, P=0.46					

Figure 1. ROC curve for HBV viral load with HBxAg

Table 3. Association of HBV viral load with HBxAg (Area Under the Curve)

Area	Accuracy	Asymptotic Sig.	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
0.768	very good	0.000	0.665	0.871

Discussion

Globally, there were an estimated 248 million persons with chronic HBV infection in 2010 and approximately 686,000 deaths were attributed to complications associated with chronic HBV infection in 2013 [3]. Chronic HBV infection remains the most important risk factor for HCC worldwide, with the level of viremia being the number one risk factor [4].

In this study, 11 out of 25 patient had complications has chronic active hepatitis in whom HBV viral load more than $4.9194*10^4$, this agrees with a study suggested that HBV-DNA levels of 10^4 copies/ml or more are the strongest predictor of future cirrhosis or HCC risk, regardless of HBeAg status and serum alanine transaminase (ALT) levels at baseline [8]. A study of Bárcena Marugán and García Garzón (2009) [9], found that if patients have high DNA levels and normal ALT, without other unfavorable prognostic factors, it is advisable to follow the patients and not to treat them, because serum DNA levels are a prognostic factor, and contribute to define the phase of CHB infection, the treatment indication, and allow an assessment of the efficacy of antiviral therapy. High levels of HBV DNA are an independent risk factor for cirrhosis and HCC in Asia.

In this study, HBxAg has positive correlation R=+0.5, P<0.000 with active infection. Hepatitis Bx gene (HBx) showed progressive increase in expression with the increase in viral copy number and showed 50% expression in patients having high viral load from 41 patient with active HBV infection there was 21 positive HBxAg this suggested HBxAg as sensitive biomarker for HBV infection follow up, this high significant correlation between HBxAg and active chronic hepatitis can nominate it as a good prognostic marker for disease progression.

Previous study has shown that HBx can manipulate epigenetic mechanisms to regulate not only host gene expression in hepatocytes but also the replicative ability of HBV itself [10].

HBx can integrate into cellular DNA during chronic infection. Overexpression of HBxAg may alter signal transduction pathways of hepatocyte, and it can bind to and inactivate negative growth regulatory genes suggesting its role in hepatocarcinogenesis [11-13].

Al-Qahtani et al. study (2017) [14], supported the results of the current study in which expression of HBxAg correlates with the activity of the disease and the possibility of development of cirrhosis and fibrosis.

The HBx protein is expressed in chronic hepatitis, cirrhotic liver and HCC from individuals infected with HBV. It is localized in both the cytoplasm and nucleus, and can therefore interact with cell signal transduction pathways and transcription machinery. HBx has been reported to transactivate a variety of cellular genes. Also, HBx associates with the p53 tumor suppressor protein in vitro and in vivo, leading to p53 inhibition of its functions. Moreover, p53 inactivation by HBx has been implicated in liver carcinogenesis [15,16].

In conclusion, the presence of HBxAg could be an important indicator of the disease progression and possibility of developing complications in patients with chronic HBV.

Author contribution

Dr. Mahdi: Collection of samples, DNA extraction and running the ELISA study of HBxAg. Al-Khezrachi: making the statistical analysis and writing the results. Dr. Shamran:

Final editing of the manuscript. Dr. Al-Obaidi: Detection of HBV DNA using quantitative real time polymerase chain reaction.

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Correspondence to Dr. Asmaa B. Al-Obaidi
E-mail: asmaa.viro@gmail.com,
asmaa.viro@ced.nahrainuniv.edu.iq
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