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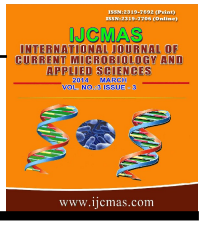

Original Research Article

Hepatitis B virus is a major culprit in hepatocytes disorders in Nigerian patients

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A B S T R A C T

Hepatitis B virus is a major public health burden in Africa, Nigeria inclusive. This study was carried out to demonstrate the presence of viral inclusion bodies of Hepatitis B virus on paraffin embedded liver biopsies. Samples were cut into 3µm sections and stained histochemically using Orcein and Phloxine –Tartrazine stains. The presence of hepatitis B surface antigen is determined by the bright red to reddish brown intrahepatic background compared to yellow background for non-infected and black to brown background in phloxine Tatraxine and Orcein methods respectively. Both methods were control using the ground glass hepatocytes staining with haematoxylin and Eosin (H/E) staining technique using light microscope. 16[72.7%] of 22 primary liver cancer cases were HBsAg positive, representing 43.3% HBsAg positive cases in 37 samples, 5(71.4%) viral hepatitis, representing 13.5%, 2(50.05%) liver cirrhosis representing 5.4% and 1(25.0%) dysplasia of the liver and represent 2.7% of the total cases respectively . Overall prevalence of HbsAg among hepatocytes disorder studied was 66.4%. Therefore from this work, there exist striking relationships between HBsAg and liver diseases and hence HBV is a major culprit in liver disorders. Screening for Hepatitis B virus in all liver disorders is pivotal in the treatment and management of liver cancer and should be included as a routine test for antenatal women.

Keywords

Liver: cirrhosis: cancer: hepatitis: histo-chemistry; stains. HBsAg: Orcein: virus: disorders.

Introduction

Hepatocellular carcinoma (HCC) is a cancer of the liver often refers to as hepatoma and accounts for 5.6% of all human cancers. It is the fifth most common cancer in men and eight most common in women worldwide and is the third leading cause of cancer related death, exceeded only by cancers of the lung and

stomach (Ihekwaba and Nwankwo, 2003). The World Health Organization (WHO) in the year 2003 reported that between five hundred thousand and one million new cases occur per year (Ihekwaba and Nwankwo, 2003). However, significant differences exist between and within countries. Most cases of HCC occur in Asia and sub-Saharan Africa. In Africa, the highest incidence was documented among the Bantu males in Mozambique (Daniele et al., 2004). In Nigeria, the prevalence of HCC increases as one migrates from the Southern rain-forest to the Northern savanna (Ihekwaba and Nwankwo, 2003; Olubuyide et al., 1986; Daniele et al., 2004). A prevalence of 0.4% was recorded in Port Harcourt south-south of Nigeria. In Ibadan, South-western Nigeria and Maiduguri, North-east of Nigeria, incidence of 4.91and11.2 per 1000 patients per year has been documented respectively.This difference in prevalence closely mirrors the HBs Ag carrier status in these geographic regions.The incidence of HCC is increasing both in the developed and developing countries (Seleye-Fubara and Jebbin, 2007). This has been attributed to the rising prevalence of its risk factors; alcohol consumption, non-alcoholic fatty liver disease (NAFLD) associated with type 11 diabetes and obesity and especially chronic hepatitis B and C infection. Despite the advances in treatment, the prognosis of HCC is still poor. This is more so in developing country like Nigeria where late presentation coupled with paucity of diagnostic and interventional facilities have rendered the tumor untreatable. This is in contrast to what obtains in the developed countries where diagnosis of the tumor in the early stages makes institution of intention-to-cure therapies possible with 5-year survival approaching 70-80%.Hepatitis B virus abbreviated HBV is specie of the genius orthohepadnavirus which is likewise a part of the Hepadnaviridae family of viruses and the virus causes the disease hepatitis. (Alberti and Caporaso, 2011; Parkin, 2011). Hepatitis B virus is a potentially life threatening liver infection globally. It is a major global health problem and the most serious type of viral hepatitis (Henry et al., 2002). It can cause chronic liver disease and put the population at a high risk of death from cirrhosis and cancer of the liver (Parkin, 2011), liver cancer cause by hepatitis B virus is among the three cause of death from cancer in men and major cause of cancer in endemic region (Buendia, 1992).

Epidemiology

Hepatitis B (HBV) infection is a major global public health problem. In 2004, estimated 350million individuals were infected worldwide. National and regional prevalence ranges from over 10% in Asia to under 0.5% in the United States and northern Europe. In japan, 2-7% of the population is chronically infected; the disease is predominantly spread among children. In high prevalence areas such as China and South East Asia, transmission during childbirth is most common, although in other areas of high endemidity such as African transmission during childhood is a significant factor (Alter, 2003). The prevalence of chronic HBV infection in areas of high endemicity is at least 8%. Routes of infection include vertical transmission (such as through childbirth), early life horizontal transmission (bites, lesions and sanitary habits) and adult horizontal transmission (sexual contact, intravenous drug use) Custer et al., 2004; Gust, 1996). It also occurs through the blood, saliva, semen,

vaginal secretions, menstrual blood, needle sticks, sharp instruments, sharing items (razors, toothbrushes). In low prevalence areas such as the continental United States and Western Europe, infection, drug abuse and unprotected sex are the primary methods (Redd et al., 2007). Infants may also develop the disease if they are born to a mother who has the virus. Infected children often spread the virus to other children if there is frequent contact or a child has many cuts and to a lesser extent, perspiration, breast milk, tears and urine of infected individuals Gust, 1996; Lok, 2003). The blood transfusions were once a common route of transmission but improved diagnostics tests and progressively boarder's screening for HBV infection in recent years such as occurred in Latin American countries from 1994 to 1997, has dramatically reduced the risk of acquiring HBV infection through transfusion (Schmunis et al., 2001). In 2010 china has 120million infected people, according to WHO an estimated 600,000 people die every year related to the infection (World Health Organization, 2012a,b). Nosocomial transmission from patient accounts for a substantial disease burden in countries with inadequate infection control practices, including reuse of contaminated medical or dental equipment, failure to use appropriate disinfection and sterilization practices for equipment and environmental surfaces and improper use of multi-dose medication vials (Alberti and Caporaso, 2011).

Mechanism of action

Hepatitis b virus (hbv) primarily interferes with the functions of the liver by replicating the liver cells, known as hepatocytes. HBV virion bind to the host cells via the pre S domain of the viral surface antigen and are subsequently internalized by endocytosis. Pre S and IgA receptors are accused of this interaction. HBV-pre S receptors are primarily expressed on hepatocytes. However, viral DNA and proteins have also been detected in extra-hepatic sites suggesting that cellular receptors for HBV may also exist on extra-hepatic cells (Lai and Yuen, 2007). During HBV infection, the host immune response causes both hepatocellular damage and viral clearance. Although the innate immune response does not play a significant role in these processes, the adaptive immune response, particularly virus specific cytotoxic T lymphocytes (CTLs) contribute to most of the liver injury associated with HBV infection. CTLs eliminate HBV infection by killing infected cells and producing anti-viral cytokines which are then used to purge HBV from viable hepatocytes (Vandamme and Van-Herck, 2007). Although liver damage is initiated and mediated by the CTLs, antigen non-specific inflammatory cells can worsen CTL induced immunopathology and platelets activated at the site of infection may facilitate the accumulation of CTLs in the liver (Locarnini, 2004).

Studies shows that a wide range of the risk ratio for liver cancer in chronic HBV carriers (5 folds to 148 fold risk increase, (International Agency for Research on Cancer, 1994; Parkin, 2011) a meta-analysis produced a risk ratio of 20 for people in area where prevalence of infection is low, (Lévy et al., 2002). Tumors tend to arise after chronic HBV infection and the greatest majority shows evidence of clinically integrated HBV DNA sequence in the tumor cells. Such evidence strongly suggests a causative role of HBV in liver carcinogenesis, but the precise mechanism through which HBV

acts is still not understood. In most cases the HBV genome is not integrated near cellular oncogenes and although some HBV proteins have interesting effects on cell growth in the laboratory, the virus does not consistently express those viral proteins when it is presents on the tumor cells. (Libra et al., 2005; Cho et al., 2011). A more likely scenario is that many virus infected liver cells are destroyed as a result of immunological attack than the virus than by the virus replication per se, such damage stimulates the remaining cells to grow and divide thereby increasing the risk of genetic accident. In addition subsequent infection of regenerating cells can lead to chance integration of viral DNA into the genome further promoting cell genomic instability. Together these effects would enhance the chances of HBV infected liver cells to accumulate series of genetic changes necessary for it malignant transformation. This work is aim at establishing the relationship between Hepatitis B virus and liver disorders in Nigerian subjects.

Materials and Methods

Study area

Calabar is the capital city of Cross River state Nigeria, West Africa. Cross River state is located in the south-south geo-political zone of Nigeria. The populace is mainly urban and inhabitant engages in civil service, fishing and oil exploration. The teaching hospital is located in one of the city metropolis providing services for the entire state and equally serves as a referral center. Approval was obtained from the Ethics and research committee of the University of Calabar Teaching hospital Calabar, Cross river state, Nigeria.

Sample collection

The specimen used in this research was obtained from patients who had undergone liver biopsy in university of Calabar teaching hospital, Calabar Nigeria. Samples were clinically diagnosed and histologically confirmed of having primary liver cancer, viral hepatitis, dysplasia of the liver and liver cirrhosis. Non pathologic post mortem samples were stained with haematoxylin and Eosin (H/E) and both Shikata Orcein and phloxine Tartrazine which serves as negative control and positive slides for HBsAg serves as negative control.

Preparation of sections

The paraffinized liver tissue blocks were sectioned were obtained from the department of Histopathology department. The tissue block was then section into 3um ribbons (thickness) using Leica microtome (LEICA RM2125 RTS). Sections were floated (using 20% alcohol) in water bath maintained at a temperature range of 37-42 degree centigrade. The desired sections were collected using Mayer egg albumin glass slides. The sectioned were dried on a hot plate at a temperature of Five degree centigrade below that of the paraffin wax. Sections were stained in haematoxylin and Eosin to demonstrate the tissue structure and in modified Orcein stain (shikata, 1974) and Pholxine –Tartrazine (Lendum 1947) techniques to demonstrate viral inclusions (John Bancroft and Marilyn, 2008). Sections were deparafinized and hydrated through grades of alcohols (ascending order) and oxidize with acid permanganate for 5mins, bleached with 1.5% aqueous oxalic acid for 30mins. Rinsed in water for 5mins and then in 70% alcohol for 2minutes, stained in Orcein solution at 37 degree centigrade for 90

minutes, rinsed in distilled alcohol and examined microscopically to determine desired intensity of staining, rinsed in cellosoluse and stained in Tartrazine solution for 2minutes, rinsed again in cellosoluse, clear and mount. Sections were air dried, mounted with DPX and viewed using light Olympus microscope. This work is aim at detecting the presence of (if any) viral inclusion bodies (HbsAg) in clinically queried patients and histologically confirmed liver disorders. .

Statistical analysis

Data were expressed as mean, using T-test for independent groups. Value considered significant at $p \leq .05$ and insignificant at $p \geq .05$. Statistical analysis was done using SPSS version 11.0 programs.

Results and Discussion

Table 3.1 shows results obtained from of liver biopsy stained with Orcein shikata method and phloxine-Tartrazine method for the demonstration of viral inclusion. The observed staining result shows that twenty-four (24) primary liver disorders were positive for Hepatitis B surface antigen (HBsAg) using both stains while thirteen (13) cases were negative. Table 3.2 shows the percentage distribution of HBsAg among the cases studied. Primary liver cancer was the hepatocyte disorder representing 22(59.45%) of the cases and 16(72.7%) were HBsAg positive. This followed by viral hepatitis 5(71.4%), liver cirrhosis 2(50.0%), and liver dysplasia 1(25.0%). Overall number of and percentage distribution of HBsAg positive cases out of the 37 cases studied were 24 representing 64.8% while the remaining were negative for both staining technique.

Hepatocellular carcinoma is one of the most common malignancies in the world. Its prevalence is determined by that of its risk factors particularly viral hepatitis B. Therefore, particularly high rates are found in sub-Saharan Africa where HBV is endemic. In Nigeria, it is the commonest cause of cancer in the medical wards and the most common cause of cancer-related death in middle aged and elderly Nigerians (World Health Organization, 2008; Parkin et al., 2002). The prevalence of HBsAg among the HCC patients in this study compares with 37.6% reported in Port Harcourt but lower than and 67% reported in Maiduguri[28-30]. This may be explained by the increasing prevalence of hepatitis B virus as one migrates from the Southern delta towards the Northern savanna of Nigeria (Solanke and Olubuyide, 1990; Nwosu et a., 2001). There has been growing recognition of Hepatitis B surface antigen in primary liver diseases (Parkin, 2011) with eight out of every ten primary liver cancer attributed to hepatitis B Virus infection. HBV DNA sequence in tumor cells strongly suggest a causative role for HBV in liver carcinogenesis, but the exact mechanism through HBV act is still not understood. Yet an accepted scenario been that many virus infected liver cells are destroyed as a result of immunological attack than virus per se and such stimulate the remaining cells to grow and divide, thereby increasing the risk of genetic accident (Cho et al., 2011). The characteristic ground–glass appearance which has affirmed to the accumulation of HBsAg in the rough and smooth endoplasmic reticule of the hepatocytes (Cho et al., 2011; Aoki et al., 1982) this actually inspired the use of histochemically techniques to demonstrate it prevalence in already diagnosed liver cancers.

The basis of the histochemical staining technique for HBsAg is that of Shikata

Table.3.1 Phyloxine and orcein stains for hepatitis b surface antigen

Types	CASES	%POSITIVE	% NEGATIVE
Liver cirrhosis	4	2(50.0)	2(50.0)
Dysplasia	4	1(25.0)	3(75.0)
Viral hepatitis	7	5(71.4)	2(28.6)
Primary Liver cancer	22	16(72.7)	6(27.3)

Table.3.2 % distribution of hepatitis b surface antigen among cases examined.

LIVER DISORDER	% NO OF CASES	% HBsAg (positive)
Cirrhosis	4(10.8)	2(5.4)
Dysplasia	4(10.8)	1(2.7)
Hepatitis	7(18.9)	5(13.5)
HCC	22(59.5)	16(43.2)
Total	37(100.0)	24(64.8)

Orcein method. It employs preferential selectivity for disulphide (-s-s-) bounds linkage predominate in HBsAg coat (Nayak and Sactideva, 1975; John Bancroft and Marilyn, 2008) while Phloxine- Tartarzine methods employ the fact Viral infected hepatocytes to retain the stain during differentiation (John Bancroft and Marilyn, 2008). The success of methods is largely dependent on the quality control of the staining medium. The method relies also on permanganate oxidization of proteins to sulfate residue that react with Orcein. The cytoplasm staining pattern (intrahepatocytes) observed with both viral staining techniques are recognized by their good contrasts staining ability. However at present Shikata Orceien method is more specific for detection of HbsAg in liver tissue when compared to phloxine-Tartraxine method. In view of the research six in every ten cases of hepatocytes disorder is attributed to hepatitis B virus. From the study, HBsAg marker occurs mostly middle age individuals and mostly most frequently in primary liver cancer and viral hepatitis with percentages of 72.7%, 43.3% and 71.4%, 13.5%. (Table 1 and 2). Also the distribution of HBsAg in liver dysplasia (25.0%, 2.7%. table 1 and 2) respectively, is assumed to be low in occurrence compared to other HBV associated liver disorder. Out of 37 cases studied for the prevalence of HbsAg 24 were positive for HbsAg (6:10) ratio. The distribution of HBsAg in these disorder are evaluated from a mean of 5.47 and an acceptance region of $3.21<\mu<7.73$, $r = 3$, $p<.05$, $0\neq0$). HBsAg distribution is assumed to increase by occurrence in liver disorder in the following order dysplasia of liver cells<liver cirrhosis<viral hepatitis<liver cancer among the thirty seven cases examined a clear indication of its culprit.

Since liver cancer develops quietly, usually without symptoms, patients with chronic HBV should be screen for liver cancer. Mass screening of the population, youths who are more prone to the risk

factors is necessary to preventive outbreak. Also mass education on the knowledge and prevention of hepatitis B virus in the country, continent and the world should be intensified. Therefore from the strength of this work Orcein and shikata methods are still very vital in the detection of viral particles in developing and underdeveloped countries.

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