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Original Article

Molecular Analysis of HBV in Histologically Confirmed Hepatocellular Carcinoma in a Tertiary Hospital in Ghana

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Abstract

Background: Hepatocellular carcinoma (HCC) is a leading cause of death in Africa. Viral hepatitis B is a leading cause of hepatocellular cancer in Ghana and most African countries except Egypt where hepatitis C virus is more prevalent. This study aims at reviewing the histopathological patterns of HCC and its association with hepatitis B virus in our environment.

Methodology: Demographics and histological diagnosis were retrieved from the surgical daybook and archival FFPE tissue samples with histopathologically confirmed HCC were used for this study. Sections (10µm) were taken from the tissues and digested to obtain DNA lysates. The DNA lysates were used in polymerase chain reaction (PCR) to determine the prevalence of HBV in the biopsies.

Result: Of the 24 confirmed cases of HCC seen in the 5-year period, there were 17 males and 7 females with M:F ratio of 2.4:1. The mean age of our patients was 39.92 ± 1.98 years with age range 13-85 years. 50% of the cases were moderately differentiated while 25% each were well and poorly differentiated. Out of the 24 archival HCC biopsies screened, HBV DNA PCR amplification was achieved in 11 (45.83%) after the restriction fragment length polymorphism PCR reaction. Out of the 24 archival HCC biopsies screened, HBV DNA PCR amplification was achieved in 11 (45.83%) after the restriction fragment length polymorphism PCR reaction. Eight of the 11 cases were found in the male and 3 in females. Of the 11 (45.83%) samples that were positive for HBV DNA, 3 were above 40 years and 8 were 40 years and younger.

Conclusion: The overall prevalence of HBV DNA in our study was 45.83% and a greater proportion seen in ≤ 40 years. This suggests that most of our patients are infected with HBV early in life in our environment.

Keywords: Hepatitis B Virus; Hepatocellular Carcinoma; Formalin Fixed Paraffin Embedded Tissue; Polymerase Chain Reaction.

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Quick Response Code:

Introduction

The liver plays important role in detoxification, biosynthesis, digestion, energy, and metabolism. Hepatocellular cancer (HCC) accounts for most of the primary liver cancers. Liver cell damage can be caused by a wide variety of infection, drugs, chemicals, and toxins [1]. Hepatitis is a liver disease which results in inflammation and swelling of the liver. It is mainly a viral infection but could be caused by autoimmune diseases, drugs and other toxins and metabolic diseases like haemochromatosis, Wilson disease and α-1antitrypsin deficiency. Viral hepatitis is a worldwide infection and a global health problem. Viral hepatitis B and C and D in association with B are the major types associated with liver cancer [2].

Hepatitis B and C are transmitted through blood and body fluid contact contaminated by the hepatitis B virus (HBV) and hepatitis C virus (HCV) respectively. Recent studies have shown and estimated that 1.25 million people in the United States have chronic hepatitis B and C and 350million people worldwide live with the disease while many more are unaware, they have it [3]. Hepatitis B virus belongs to hepadnaviridae family and has eight genotypes namely: A,B,C,D,E,F,G,H which tend to cluster geographically with genotype A and E being predominant in Africa[4,5]. Hepatitis C on the other hand belongs to the family flaviviridae and a common cause of liver disease worldwide [6]. HCV has seven genotypes and a number of subtypes [7,8]. Hepatitis D virus (HDV) is a rare form of hepatitis but very severe and occurs in conjunction with hepatitis B infection. HDV requires the presence of another virus to replicate, notably HBV. The different types of viral hepatitis all cause acute hepatitis, but HBV and HCV can result in chronic hepatitis leading to liver cirrhosis and fibrosis to hepatocellular cancer [9,10,11].

Liver cancer is one of the most common visceral malignancies worldwide and is estimated to be responsible for more than a million deaths annually [12]. Hepatocellular carcinoma (HCC) by anecdotal reports has its largest burden in Africa especially Sub-Saharan part of the continent [13,14]. It is a leading cause of death in Africa. Hepatocellular carcinoma is a leading cause of cancer-related death in many African countries because of the combination of high incidence and high cancer-specific mortality [12,13]. Viral hepatitis B is a leading cause of hepatocellular cancer in Ghana and most African countries except Egypt where hepatitis C virus is more prevalent [14]. In addition to its high incidence, hepatocellular carcinoma is diagnosed at an earlier age and at more advanced stages in Africa than in the rest of the world[15]. This study aims at reviewing the pathological characteristics of hepatocellular carcinoma in our centre, to grade the tumours for prognostication and to determine the presence of HBV DNA in FFPE tissue blocks using polymerase chain reaction.

Methodology

Study design.

The proposed research is a retrospective study design with a laboratory analysis of archived primary HCC tissue samples from 2009-2014. Twenty-four HCC paraffin- embedded tissue samples with histopathologically confirmed cases from the pathology department of our hospital were used. Data regarding the age, sex, histological diagnosis, and level of differentiation for each sample were recorded.

Molecular analysis

Tissue sectioning for histological analysis and genomic DNA extraction

Paraffin embedded HCC and liver disease tissue samples were retrieved from archivers at the pathology department of KATH. These blocks were confirmed with the original diagnosis. The paraffin embedded tissues were trim, and microtome was used to take 10µm and 5µm sections of each tissue. The 10µm was then placed into a sterile 2.0ml micro centrifuge tube. The tubes were used for the HBV-DNA detection and genotyping. The tissues in the micro-centrifuge tubes were digested in 250µl of digestion-buffer by incubation for 16 hours at 55ºC on a heat-block. The digestion buffer consisted of the following components: 200µg/ml Proteinase K (Sigma, USA), 50mM Tris-HCL (pH 8.5), 1mM EDTA, and 0.5% Tween-20. After the 16 hours incubation, the proteinase K was inactivated at 100˚C for 5 minutes on a heat-block. After cooling of the samples to room temperature, the samples were then centrifuged at 13000 rpm for 5 minutes in a micro centrifuge. The residues containing lysate were pipetted out and the supernatant containing paraffin was kept. The digested product of each sample was aliquot into different tubes and stored at -20˚C freezer for future use. The residue realized after the processes was the DNA extract that was used as a source of DNA template for the PCR analysis following the manufactures protocol[16,17].

HBV-DNA detection method

HBV-DNA detection was carried out by a restriction fragment length polymorphism18. To analyze the quality of target DNA for polymerase chain reaction (PCR) testing, the HCC specimens was screened with beta-globin gene-specific primers prior to HBV DNA testing. A single consensus forward primer GP-E6-3F {GGG (A/T) G(G/T) (G/T)AC TGA AAT CGG T}and two consensus back primers GP-E7-5B and GP-E7-6B {CTG AGC TGT CA(A/G) CTA ATT GCT CA and TCC TCT GAG T(C/T)G (C/T)CT AAT TGC TC} was used for the general primer PCR. The PCR reaction mix of 50µl contained 5µl of each DNA sample which was pipetted into different tubes; 25µl of the master mix was added to each sample. The master mix was made up of 10x PCR buffer, 15µl MgCl2, dATP, dGTP, dTTP, dCTP, and Taq polymerase. 5ul each of HBV forward primer and reverse primer was added to each sample and 10µl of nuclease free water was added to each sample making a total volume of 50µl of each sample. Samples were put into the thermocycler for incubation. The thermocycler was programed in this process; an initial denaturation at 94ºC for 3min which was then followed by 40 cycles of amplification which included another denaturation at 94ºC for 45seconds, annealing at 53ºC for 60seconds, extension at 72ºC for 90seconds which was prolonged by 3 cycles were done which was then followed by a final extension at 72ºC for 7minutes and holding at 4ºC17. PCR products were mixed with1µl (5u) of Tsp5091, 1.5µl of10x buffer and 2.5µl of water and then incubated at 65ºc for 3hours. In a separate reaction, PCR product (10µl) was mixed with 0.5µl (5u) of Hinfi, 1.5µl of 10x buffer and 3µl of water and incubated at37ºc for 3 hours.

Analysis of amplification products

The amplification products for HBV were analyzed by gel electrophoresis on 2% agarose gel and stained with 0.5µg/ml ethidium bromide. Five microlitres of each sample was added to 2µl of orange G (5X) gel loading dye for the electrophoresis. Hundred base pair DNA molecular weight marker (Sigma, MO, USA) was run alongside the PCR products. The gel was prepared and electrophoresed in 1X TAE buffer using a mini gel system at 100 volts for 45minute and was optically visualized and photographed over a UV trans-illuminator.

Human β globulin PCR

The DNA extracts were analyzed for the presence of human genomic DNA by human β-globin DNA PCR using the primers; PCO3+ and PCO4+17,19. This PCR analysis served as quality control to ascertain the presence of human genomic DNA in the extract and the wholesomeness of the DNA in the DNA tissue extract for PCR. The PCR condition was as by de Roda Husman et al18 with few modifications using Techne PCR machine (Techne Incorporated, USA). For a total volume of PCR reaction mix of 25µl the reaction conditions was as follows; 3µl of 10X PCR buffer (Biopioneer, Co, USA), 1.5mM MgCl2 (Biopioneer Co, USA), 200µM of each of the four oligonucleotide triphosphates (dNTPs) (Sigma, Co, USA), 25picomoles (pmol) of each consensus primers PCO3 and PCO4 and 0.625units of Taq Polymerase enzyme (Biopioneer Co, USA) and 1µl or 4µl 4of the DNA lysate; the tissue digested product (source of HPV DNA template). Nuclease free water (Promega Co, USA) was used to make up the volume to 25µl. A master mix was made without the DNA template, vortex to mix and then pulse centrifuged for 30 seconds before aliquoting out into the samples in the PCR tubes. The negative control (25µl of the master mix) and positive control were also taken through the same PCR reaction conditions as per samples. The PCR cycling conditions were as follows; 95ºC for 4minutes followed by 40 cycles of 95ºC for 1min, 55ºC for 2mins, 72ºC for 1.5mins and final elongation step of 72ºC for 4mins19. After the reaction, 10µl of the PCR product was then separated on 2% agarose gel (Biopioneer Co, USA) in 1X TAE buffer (Biopioneer Co, USA) through gel electrophoresis at 80 volts (Labnet International, Power station 300) using 2µl of blue/orange DNA loading dye (6X) (Promega Co, USA) and stained with 0.5µg/ml ethidium bromide (Life Technologies Co, USA). Hundred base pair nucleotide sequence molecular size marker (Sigma Mo, USA) was run alongside the PCR products on the gel. The gel was photographed using UV-illumination (UVIsave gel documentation system, model GAS9200/1/2/3, Version 12) and the picture was then analyzed after the end of electrophoresis.

Histopathological diagnosis

The 5µm section of each tissue sample was placed on a slide for the histopathological diagnosis which was then by staining each sample with H&E. New microtome blade was used for each sample.

Statistical analysis

The data obtained was analyzed by using SPSS version 21 and results were put in tables and graphs. Statistically significant level was set at 0.05 and proportions and percentages were used to summarize the data for qualitative variables whiles means and standard deviations were used to summarize quantitative variables. Chi-square was used to check for associations between the presence of HBV and subject characteristics (age and sex) as well as the presence of HBV with cancer grade. Regression analysis was carried out to check for relations and where appropriate McNamara test was used to compare proportions.

Ethical approval

Ethical clearance was obtained from KATH/KNUST review committee for review and approval before conducting the studies.

Results

Demographic and clinical data of study subjects

Table 1 shows the demographic data of the HCC cases studied. The twenty-four HCC cases studied included 17(70.83 %) males and 7 (29.17%) females with male to female ratio 2.4:1 and age range 18 to 85 years. Histological grading in table 2 showed that 6 (25%) tumours were well differentiated (G1grade), 12 (50%) were moderately differentiated (G2), 6 (25% were poorly differentiated (G3).

Human β-globulin PCR analysis

Figure 1 shows an electrophoregram of PCR amplification of human β-globulin gene in the tissues. The DNA in the resultant human β-globulin PCR product of the 24 tissue samples gave bands in the 100bp region, indicative of the correct amplicon size of β-globulin gene.

The overall prevalence of Hepatitis B Virus in the study sample

Out of the 24 archival HCC biopsies screened, HBV DNA PCR amplification was achieved in 11 (45.83%) after the restriction fragment length polymorphism PCR reaction (Figure 3)

Hepatitis B Virus status and gender

Out of the 24 cases studied, 11 (45.83%) of the 24 studied samples were positive for HBV DNA of which 8 were males and 3 were a females 13 (54.17%) negative of which 9 were males and 4 were females. (Table 3, Figure 2). Males were found to be 1.051 times more likely to be infected with HBV than females. However, the association between gender difference and HBV infection was not statistically significant (OR=1.185; 95% CI=0.201-6.987; p = 0.341).

Hepatitis B Virus status and age

The mean age of our cases was 39.92 years ± 1.98 SD years (modal age 30-39 years; range 13 to 85(72) years). Of the 13(54.17%) HBV negative cases, 7 were 40 years and below, 6 were above 40 years. And of the 11 (45.83%) samples that were positive for HBV DNA, 3 were above 40 years and 8 were 40 years and below (Table 4). The 40 years and below age group were 1.4 times more likely to be infected with HBV than those in the above 40 years age group. However, there was no statistically significant association between age difference and HBV infection (p value = 0.217; OR=1.4; 95% CI=0.272-2.510)

Hepatitis B virus status and tumour grade

As seen in Table 5, out of the 11 HBV DNA positive cases detected, 4 were well differentiated; 5 were moderately differentiated and 2 were poorly differentiated. Considering the 13 HBV negative HCC cases identified, 2 were well differentiated, 7 moderately differentiated, 4 poorly differentiated.

Table 1: Demographic distribution of study population

Parameter	Frequency	Percentage
Sex		
Male	17	70.83
Female	7	29.17
Total	24	100.00
AGE Group		
10-19	3	12.5
20-29	5	20.8
30-39	7	29.2
40-49	3	12.5
50-59	2	8.3
60-69	2	8.3
70-79	0	0
80-89	2	8.3
Total	24	100.00

Table 2: Histological grading of tumour

Tumour grade	Frequency	Percentage
G1	6	25
G2	12	50
G3	6	25
Total	24	100.00

Table 3: Hepatitis B Virus status and gender

Gender	HBV+ (%)	HBV- (%)	Total
Male	8 (33.33)	9 (37.50)	17(70.83)
Female	3 (12.50)	4 (16.67)	7 (29.17)
Total	11 (45.83)	13 (54.17)	24 (100)

Table 4: Hepatitis B virus status and age

Age grouping (years)	HBV+	HBV-	Total
≤ 40	8(33.33)	7(29.17)	15(62.50)
> 40	3(12.50)	6(25.00)	9 (37.50)
Total	11(45.83)	13 (54.17)	24 (100)

Table 5: Hepatitis B virus status and tumour grade

Tumour grade	HBV+ (%)	HBV- (%)	Total
G1	4 (16.67)	2 (8.33)	6 (25.00)
G2	5 (20.83)	7 (29.17)	12 (50.00)
G3	2 (8.33)	4 (16.67)	6 (25.00)
Total	11 (19.23)	13 (80.77)	24 (100)

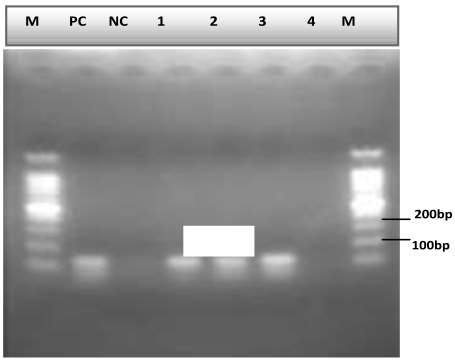

Figure 1: An electrophoregram of human β globulin PCR products. The 100bp fragment corresponding to amplified human β globulin DNA. Lane M: 100bp molecular size marker; NC: negative control (no DNA); PC: positive control (human DNA purified from blood); 1, 2 and 3: human β globulin DNA positive samples respectively.

Figure 2: An electrophoregram of Hepatitis B virus PCR products. Lane M: 100bp molecular size marker; Lane 2: negative control (no DNA); Lane 1: positive control (DNA); Lane 3-6; HBV DNA positive samples; Lane 7; HBV DNA negative samples.

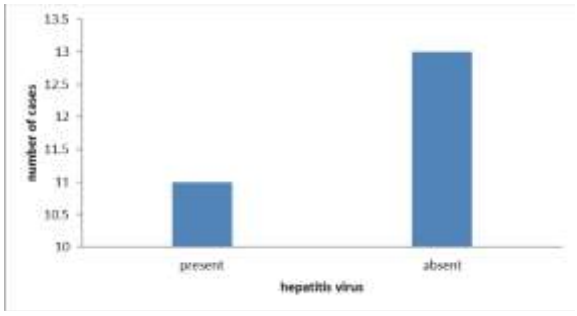

Figure 3: A bar graph of the frequency of HBV negative and HBV positive HCC cases in the study population.

Discussion

This study looked at the association of HBV with HCC using FFPE of histologically confirmed cancer by PCR method. It is worth noting that all the samples were positive for human β-globulin DNA hence the DNA extracts from the FFPE archival HCC tissue samples used in this study were suitable for PCR analysis [19].

Viral hepatitis is a major health problem worldwide with greater burden in the developing countries [20]. It is a major risk factor to primary liver cell carcinoma [21,22]. The mortality and morbidity of liver cancer in our environment is on the increase due to inadequate diagnostic and management wherewithal [13,14]. Most of the patients with liver cancer in our environment present with higher grade which are generally more aggressive and hence dismal prognosis. In our study, about 75% of our cases were high grade tumours consistent with most studies worldwide [23,24].

The overall prevalence of HBV DNA in our study was 45.83% with a slightly higher occurrence in males. Higher prevalence of HBV infection was found in males where males were found to be 1.1 times more likely to develop HCC than females in the study but there was no statistically significant association between gender difference and HBV infection (p value=0.341; 95%CI=0.629-1.753). Similar findings were found in the study of Jang SY, et al[25] in South Korea where gender differences

in HBV infection was 1.19 with slight male preponderance. The study of Baig [26] in 2009 showed a more preponderance of males having HBV infection and subsequent development of HCC than females (M:F ratio of 3.8:1). He suggested that there may be an influence of estrogen in the protection and defense of hepatic cells against the development of chronic liver disease. A report by Wiredu and Armah [13] found out that in males, the highest mortality of cancer was liver cancer (21.15%) compared to all cancers that affect males; and of the liver cancer, the relative frequency (RF%) for males was 21.3 compared to females of relative frequency (RF%) of 10.9. This probably explains why more males HBV infection had than females in this study and why more males had HCC. Also, a study on the prevalence of asymptomatic HBV in children in Ilesha, Nigeria found a slight female preponderance of 15.4% compared to male of 12.7% [27].

A study conducted by Ding et al [7] in 2003 on the prevalence of HBV DNA in China found 164 (72.6%) out of 226 tested, positive for HBV DNA and they concluded that HBV infection was a primary cause of liver cancer in China. In a study by Paterlini [28] in 1990, antibodies to the surface and core antigens of HBV were detected in 10 out of 23 patients tested. HBV DNA sequence was detected in 13 out of the 28 patients including 8 of the 10 with HBV antibodies and 6 of the 13 without HBV serological markers. This showed that transcriptionally active HBV genomes are present in various geographical areas among patients with liver cancer consistent with an aetiologic role for the virus in the development of these tumours[28]. These studies corroborated our findings in the present study.

Eight out of the eleven positive HBV DNA samples were 40 years and younger. This shows that HCC occur in younger age group in our region compared to the developed world [14,15,29]. This may be a result of vertical transmission of HBV or the acquisition of the virus early in childhood which is relatively common in sub-Saharan Africa due largely to lack of screening of pregnant mothers and vaccination of infants [30,31,32].

A greater percentage of our cases were high grade tumours (Grade II/III) (n=18/24). This portends aggressive tumour, greater metastatic potential, and poor prognosis. Numerous studies from developed and developing countries show similar finding [14,15]. Also all the HCC from our study were associated with cirrhosis. This affirms the pathogenesis of primary liver cancer from HBV being associated with cirrhosis in a greater number of cases.

Conclusion

Our study showed that HCC in our environment is usually high grade with the potential of being aggressive and with increased morbidity and mortality. The overall prevalence of HBV DNA in our study was 45.83% and a greater proportion was seen in subjects younger than 40 years. This suggests that most of our patients are infected with HBV early in life in our local environment. We therefore recommend that all pregnant mothers should be screened for HBV; and HBV vaccination should be added to the national programme on immunization.

Declaration/Acknowledgement

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The excel data used to support the findings of this study may be released upon application to the Committee on Human Research, Publication and Ethics of School of Medical Sciences/Komfo Anokye Teaching Hospital, at Block J, School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

References

1. Kalra A, Tuma F. Physiology, liver. StatPearls [Internet] Publishing; Treasure Island (FL): Dec 18, 2018.
2. Marcellin P, Dusheiko G, Zoulim F, Esteban R, Hadzi-Yannis S, Lampertico P, et al. European association for the study of the liver. Journal of hepatology 2009;50: 227–242.
3. Ohfuji S, Fukushima W, Tanaka T, Habu D, Tamori A, Sakaguchi H, et al. coffee consumption, and reduced risk of hepatocellular carcinoma among patients with chronic type c liver disease: a case-control study. Hepatol Res 2006 ; 36 :201–208.
4. Orito E, Ichida T, Sakugawa H, Sata M, Horiike N, Hino K, et al. Geographic distribution of hepatitis b virus (hHBV) genotype in patients with chronic HBV infection in Japan. Hepatology; 2001; l:34(3).
5. Wong GL, Chan HL. Molecular virology in chronic hepatitis b: genotypes. Hosp Med. 2005; 66:13-6
6. Ning Wang, Xue –Qin Gao, Jjin-Xiang Han. Simultaneous detection of HBV/HCV by multiplex PCR normalization. World Journal of Gastroenterology 2004.10, 2439-2443.
7. Ding X, Gu H, Zhong ZH, Zilong X, Tran HT, Iwaki Y, et al. Molecular epidemiology of hepatitis virus and genotypic distribution of hepatitis B and C virus in Harbin China. Jpn J infect Dis 2003; 56:19-22.
8. EASL clinical practice guidelines. Management of Hepatitis C Virus infection. J Hepatol 2011; 55:245–264.
9. Wong GL, Chan HL, Yiu KK, Lai JW, Chan VK, Cheung KK, et al. Meta-analysis: the association of hepatitis b virus genotypes and hepatocellular carcinoma. Hepatology 2013; 37:517-526.

10. Perz JF, Armstrong GL, Farrington LA, Hutin YJF, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. J Hepatol 2006; 45:529-538
11. Sumi H, Yokosuka O, Seki N, Arai M, Imazeki F, Kurihara T et al. Influence of Hepatitis B Virus genotypes on the progression of chronic type B liver disease. Hepatology 2003; 37:19–26.
12. Globocan 2018: www.uicc.org/new-global-cancer-data
13. Wiredu KE, Armah BH. Cancer mortality patterns in Ghana: 10 years review autopsies and hospital mortality. BMC Public Health, 2006; 6:159.
14. Yang JD, Mohamed EA, Abdel Aziz AO, Shousha HI, Hashem MB, Nabeel MM et al. Characteristics, management, and outcomes of patients with hepatocellular carcinoma in Africa: a multicountry observational study from the Africa Liver Cancer Consortium. Lancet Gastroenterol Hepatol. 2017; 2:103-111
15. Yang JD, Gyedu A, Afihene MY, Duduyemi BM Micah E, Kingham TP et al. Hepatocellular carcinoma occurs at an earlier age in Africans, particularly in association with chronic hepatitis B. Am J Gastroenterol 2015; 110:1629–1631.
16. Dabic MM, Hlupic L, Babic D, Jukic S, Seiwerth S. Comparison of polymerase chain reaction and catalyzed signal amplification in situ hybridization methods for human papillomavirus detection in paraffin-embedded cervical preneoplastic and neoplastic lesions. Archives of Medical Research 2004; 35:511-516.
17. Asante DB, Asmah RH, Adjei AA, Kyei F, Simpong DL, Brown CA, Gyasi RK. "Detection of Human Papillomavirus Genotypes and Epstein-Barr Virus in Nasopharyngeal Carcinomas at the Korle-Bu Teaching Hospital, Ghana," The Scientific World Journal, vol. 2017, Article ID 2721367, 9 pages.
18. Lindh M, Anderson AS, Gusdal A. Genotypes, nt 1858 variants and geographic origin of hepatitis B virus large scale analysis using a new genotyping method. J Infect Dis. 1997; 175:1285–1293.
19. De Roda Husman AM, Snijders PJF, Stell HV, Van den Brulel AJC, Meijer CJLM, Walboomers JMM.1995 Processing of long-stored archival cervical smears for human papillomavirus detection by the polymerase chain reaction. Br J Cancer 1995; 72:412-417
20. Abdo AA, Mal-Jarallah B, Msanai F, Hersi AS, Al-Swat K, Azzam NA, Al-Dukhayil M., et al (2006). Hepatitis B genotypes: relation to clinical outcome in patients with chronic hepatitis B in Saudi Arabia. WJG 2006; 12:7014-7024.
21. Beasley RP, Hwang LY. Hepatocellular carcinoma and hepatitis B virus. Semin Liver Dis 1984; 4:113-21
22. Bosch FX, Ribes J, Cleries R, Diaz M. Epidemiology of hepatocellular carcinoma. Clin Liver Dis 2005; 9:191-211.
23. Tamura S, Kato T, Berho M, Misiakos EP, O'Brien C, Rajender Reddy K, et al. Impact of Histological Grade of Hepatocellular Carcinoma on the Outcome of Liver Transplantation. Arch Surg. 2001; 136:25-30.
24. Mise K, Tashiro S, Yogita S, Wada D, Harada M, Fukuda Y, et al. Assessment of the biological malignancy of hepatocellular carcinoma: relationship to clinicopathological factors and prognosis. Clin Cancer Res. 1998; 4:1475-1482
25. Jang SY, Jang SI, Bae HC, Shin J, Park EC. Sex Differences Associated with Hepatitis B Virus Surface Antigen Seropositivity Unawareness in Hepatitis B Virus Surface Antigen-positive Adults: 2007-2012 Korea National Health and Nutrition Examination Survey. J Prev Med Public Health 2015; 48:74-8
26. Baig S. Gender disparity in infections of Hepatitis B virus. J Coll Physicians Surg Pak. 2009; 19:598-600
27. Donbraye E, Japhet MO, Adesina OA, Abayomi OA. Prevalence of asymptomatic hepatitis B virus surface antigenemia in children in Ilesha, Osun state, South-Western Nigeria - HBV serology and Occult hepatitis. African Journal of Microbiology Research 2014; 8:2329-2331
28. Paterlini P, Gerken E, Nakajima S, Terre A, D'Errico W, Nalpas B, et al. Polymerase chain reaction to detect HBV DNA and RNA sequence in primary liver cancers from patients negative for Hepatitis B surface antigen. N Engl J Med 1990; 323:80–85.
29. Ladep NG, Lesi OA, Mark P, Lemoine M, Onyekwere C, Afihene M, et al. Problem of hepatocellular carcinoma in West Africa. World J Hepatol. 2014; 6:783-792
30. Yelemkoure ET, Yonli AT, Montesano C, Ouattara AK, Diarra B, Zohoncon TM, et al. Prevention of mother-to-child transmission of hepatitis B virus in Burkina Faso: Screening, vaccination, and evaluation of post-vaccination antibodies against hepatitis B surface antigen in newborns. J Public Health Afr. 2018; 9:816
31. Simpore J, Granato M, Santarelli R, Nsme RA, Coluzzi M, Pietra V et al. Prevalence of infection by HHV-8, HIV, HCV and HBV among pregnant women in Burkina Faso. J Clin Virol 2004; 31:78-80
32. MacLean B, Hess RF, Bonvillain E, Kamate J, Dao D, Cosimano A et al. Seroprevalence of hepatitis B surface antigen among pregnant women attending the Hospital for Women & Children in Koutiala, Mali. South Afr Med J 2012; 102:47.