Life Science Informatics Publications
Research Journal of Life Sciences, Bioinformatics, Pharmaceutical and Chemical Sciences
Journal Home page http://www.rjlbpcs.com/
RJLBPCS
ISSN 2454-6348

Original Research Article **DOI: 10.26479/2021.0702.01**

STUDY ON THE EFFECTS OF HEPATITIS B VIRUS X PROTEIN (HBx) IN HEPATOCYTES DURING INFECTION

B. A. Jinjiri*

Department of Science Laboratory Technology, Mai Idris Alooma Polytechnic, Geidam, Yobe State, Nigeria.

ABSTRACT: The HBx plasmid DNA was isolated using midi-preparation by alkaline lysis method, cell culture of HepG2 (Human liver hepatocellular carcinoma) cell line was made and transiently transfected with HBx plasmid. The protein expression of HBx is visualized under florescent microscope as the excitation of HBx is at 244nm a blue light was used for causing the excitation. The samples are collected and prepared for SDS-PAGE (sodium dodecyl sulphate polyacrylamide Gel electrophoresis) therefore, the protein of SDS-PAGE was transferred into nitrocellulose membrane for western blotting in order to check specific expression of HBx protein. The protein bands of western blotting have corresponded to 40KDa and 33KDa in both the absence and present of loading control, GAPDH (Glyceraldehyde 3 phosphate dehydrogenase) was used as a loading control. It has been observed that the HBx plasmid transiently transfected in HepG2 cell line showed its protein expression. In conclusion, HBx is responsible for protein expression and hepatocellular carcinoma (HCC) in hepatocytes during hepatitis B virus (HBV) infections.

Keywords: Hepatitis B Virus X protein (HBx), plasmid DNA, Hepatocytes, infection, cell-line.

Article History: Received: February 05, 2021; Revised: February 20, 2021; Accepted: March 01, 2021.

Corresponding Author: B. A. Jinjiri*

Department of Science Laboratory Technology, Mai Idris Alooma Polytecnic, Geidam, Yobe State, Nigeria. Email Address: babangidajinx@gmail.com

1.INTRODUCTION

Molecular cloning and DNA sequencing of the genomes of hepatitis B virus (HBV) [1] and the related woodchuck (WHV) and ground squirrel (GSHV) hepatitis viruses [1,2] revealed the conservation of four open reading frames (ORFs). Three of these ORFs could be assigned to known

viral proteins—polymerase (P), surface antigen (S) and core antigen (C). However, no known protein or function could be assigned to the smallest fourth ORF that could potentially code for a basic polypeptide of 154 amino acids. Originally called as region 5 [1], and later as region X this small ORF was postulated to have a role in viral transcription [4]. The existence of the X gene was finally established in 1985 by the cross-reaction of sera from HBV-infected patients with the recombinant hepatitis B Virus X protein (HBx) expressed in a mammalian cell line or in *Escherichia coli* [5,6]. Although X-sequences are found in both genomic and sub-genomic mRNA forms, it is encoded primarily by a 0.7-kb mRNA species [6,7]. As HBV shows extreme host tropism and cannot be grown easily in heterologous systems, structure function studies of HBx have been carried out in human hepatocyte culture

1.1 Structure of Hepatitis B Virus (HBV*)*

squirrels, tree squirrels, Peking ducks, and herons. Based on sequence comparison, HBV is classified into eight genotypes, A to H. Each genotype has a distinct geographic distribution. Three types of viral particles are visualized in infectious serum by electron microscopy. Two of the viral particles are smaller spherical structures with a diameter of 20 nm and filaments of variable lengths with a width of 22 nm. The spheres and filaments are composed of hepatitis B surface antigen (HBsAg) and host-derived lipids without viral nucleic acids and are therefore non-infectious. [8]. The infectious HBV virion (Dane particle) has a spherical, double-shelled structure 42 nm in diameter, consisting of a lipid envelope containing HBsAg that surrounds an inner nucleocapsid composed of hepatitis B core antigen (HBcAg) complexed with virally encoded polymerase and the viral DNA genome. The genome of HBV is a partially double-stranded circular DNA of about 3.2 kilobase (kb) pairs. The viral polymerase is covalently attached to the 5′ end of the minus strand.

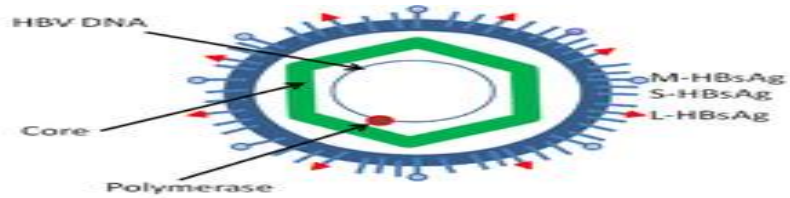

Figure 1.1: Structure of Hepatitis B (HBV)

1.2 Organization of Hepatitis B Virus

The viral genome encodes four overlapping open reading frames (ORFs: *S*, *C*, *P*, and *X*). [9] The *S*ORF encodes the viral surface envelope proteins, the HBsAg, and can be structurally and functionally divided into the pre-S1, pre-S2, and S regions. The core or *C* gene has the precore and core regions. Multiple in-frame translation initiation codons are a feature of the *S* and *C* genes, which give rise to related but functionally distinct proteins. The *C* ORF encodes either the viral

nucleocapsid HBcAg or hepatitis B e antigen (HBeAg) depending on whether translation is initiated from the core or precore regions, respectively. The core protein has the intrinsic property to self-assemble into a capsid-like structure and contains a highly basic cluster of amino acids at its C-terminus with RNA-binding activity. [10]. The precore ORF codes for a signal peptide that directs the translation product to the endoplasmic reticulum, where the protein is further processed to form the secreted HBeAg. The function of HBeAg remains largely undefined, although it has been implicated as an immune tolerate, whose function is to promote persistent infection. [11]. The polymerase (pol) is a large protein (about 800 amino acids) encoded by the *P* ORF and is functionally divided into three domains: the terminal protein domain, which is involved in encapsidation and initiation of minus-strand synthesis; the reverse transcriptase (RT) domain, which catalyzes genome synthesis; and the ribonuclease H domain, which degrades pregenomic RNA and facilitates replication. The HBV *X* ORF encodes a 16.5-kd protein (HBxAg) with multiple functions, including signal transduction, transcriptional activation, DNA repair, and inhibition of protein degradation. [12]. The mechanism of this activity and the biologic function of HBxAg in the viral life-cycle remain largely unknown. However, it is well established that HBxAg is necessary for productive HBV infection in vivo and may contribute to the oncogenic potential of HBV.

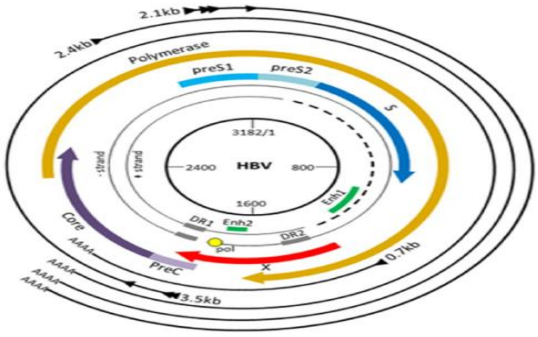

Figure 1.2: showing the genome organization of Hepatitis B Virus

1.3 HBx Open Reading Frame (ORF)

The X protein is well conserved among (mammalian) orthohepadnavirus, but absent in avihepadnavirus. Similarity of X with host cellular proteins appeared to be below or near the threshold level of detection and a crystal model of its 3D-structure is currently not available. Here, we present an *in silico* generated model of the X tertiary structure. The X model of choice is the best of the 5 alternative structures constructed by the modeling software. In docking experiments of the X structures with HBXIP or DDB1 into heterodimers, X model 1 outperforms most other models regarding the interface stability of these complexes. Amino acid residues in X proven to be critical for dimer formation with HBXIP are among the contact residues of the interface. In heterodimers of DDB1 with full-length protein X, the interfaces contain most of the H-box α-helical X residues that

were described to be involved in a DDB1/H-box oligopeptide complex. [13]. We have queried the PDB database for proteins displaying similarity with the X 3D-structure and found a striking similarity of X with members of the *MUG* family of DNA glycosylases, which are the key enzymes of the BER (base excision repair) pathway. [14]. Even the hypothetical translation product of a vestigial X reading frame in duck hepadnavirus - after restoration of stop codons into coding triplets. [14,15]. - showed a 3D-structure with significant similarity to *MUG* proteins. Protein-DNA docking experiments indicated a binding capability of X protein to an oligodeoxynucleotide that has been analyzed by X-ray in complex with E. coli *MUG* DNA glycosylase, Jiricny J [15]. From the evolutionary point of view, orthohepadnavirus and avihepadnavirus share a common protein X ancestor with orthology to DNA glycosylase.

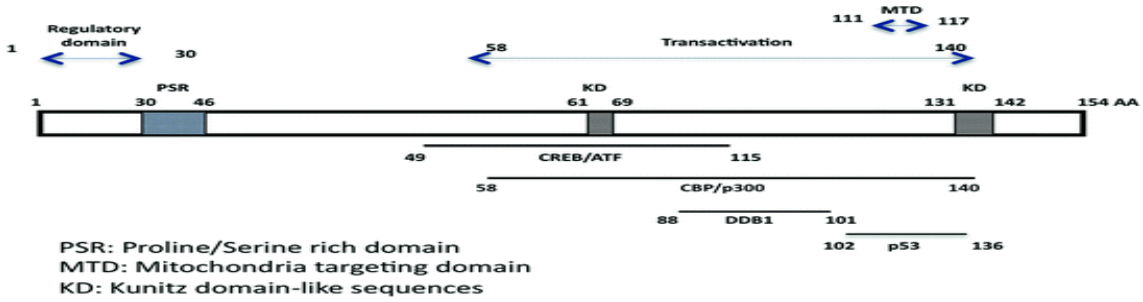

Figure 1.3: Illustrates Gene structure of HBx

1.4 Acute Infection of HBV

About two-thirds of patients with acute HBV infection have a mild, asymptomatic and subclinical illness that usually goes undetected. [16]. Approximately one-third of adults with acute HBV infection develop clinical symptoms and signs of hepatitis, which range from mild constitutional symptoms of fatigue and nausea, to more marked symptoms and jaundice, and rarely to acute liver failure. The clinical incubation period of acute hepatitis B averages 2–3 months and can range from 1–6 months after exposure, the length of the incubation period correlating, to some extent, with the level of virus exposure. [17]. The incubation period is followed by a short preicteric or prodromal period of constitutional symptoms such as fever, fatigue, anorexia, nausea, and body aches. During this phase, serum ALT levels rise and high levels of HBsAg and HBV DNA are detectable. The preicteric phase lasts a few days to as long as a week and is followed by onset of jaundice or dark urine. The icteric phase of hepatitis B lasts for a variable period averaging 1–2 weeks, during which viral levels decrease. In convalescence, jaundice resolves but constitutional symptoms may last for weeks or even months. During this phase, HBsAg is cleared followed by the disappearance of detectable HBV DNA from serum. Acute liver failure occurs in approximately 1% of patients with acute hepatitis B and jaundice [18]. The onset of fulminant hepatitis is typically marked by the sudden appearance of fever, abdominal pain, vomiting, and jaundice, followed by disorientation, confusion, and coma. HBsAg and HBV DNA levels generally fall rapidly as liver failure develops,

and some patients are HBsAg-negative by the time of onset of hepatic coma. Patients with acute liver failure due to hepatitis B require careful management and monitoring and should be referred rapidly to a tertiary medical centre with the availability of liver transplantation [19].

1.5 Chronic infection of HBV

Chronic hepatitis B has a variable and dynamic course. Early during infection, HBeAg, HBsAg, and HBV DNA are usually present in high titters, and there are mild to moderate elevations in serum aminotransferase levels. With time, however, the disease activity can resolve either with persistence of high levels of HBeAg and HBV DNA (the "immune tolerance phase") or with loss of HBeAg and fall of HBV DNA to low or undetectable levels ("inactive carrier state"). Other patients continue to have chronic hepatitis B, although some lose HBeAg and develop anti-HBe (HBeAg-negative chronic hepatitis B) [20]. The overall prognosis of patients with chronic hepatitis is directly related to the severity of disease. For those with severe chronic hepatitis and cirrhosis, the 5-year survival rate is about 50%. [21]. Among patients with evidence of chronic hepatitis (elevated ALT and inflammation and/or fibrosis on liver biopsy), many are asymptomatic or have nonspecific symptoms, such as fatigue and mild right upper quadrant discomfort. Patients with more severe disease or cirrhosis may have significant constitutional symptoms, jaundice, and peripheral stigmata of end-stage liver disease including spider angiomata, palmar erythema, splenomegaly, gynecomastia, and fetor hepaticus. Ascites, peripheral edema, encephalopathy, and gastrointestinal bleeding are seen in patients with more advanced cirrhosis. ALT and AST are often elevated but may not correlate well with severity of liver disease. Bilirubin, prothrombin time, and albumin often become abnormal with progressive disease. Decreasing platelet count is often a poor prognostic sign. Patients with chronic hepatitis may develop acute exacerbations with markedly elevated serum ALT. This scenario is more frequently described in those with HBeAg-negative chronic hepatitis B [22]. To distinguish between acute hepatitis B and chronic hepatitis B with a flare, anti-HBc IgM is a useful marker, as described in the previous section. However anti-HBc of the IgM class can be detected occasionally in patients with chronic hepatitis B with exacerbation. Alpha-fetoprotein (AFP), used as a marker for HCC, is often elevated in parallel with ALT during acute exacerbation [23]. However, it is unlikely to exceed 400 ng/mL. In patients with AFP much greater than this level, development of HCC should be suspected [24]. An estimated one-third of persons with chronic HBV infection will ultimately develop a long-term consequence of the disease, such as cirrhosis, end-stage liver disease, or HCC. The determinants of outcome of chronic hepatitis B appear to be both viral (HBV DNA levels, HBV genotype, some HBV mutation patterns) and host-specific (age, gender, genetic background, immune status).

2. MATERIALS AND METHODS

2.1. Preparation of competent cells using calcium chloride method

0.2g of Luria Bertini (LB) was poured into a falcon tube and dissolved in 10ml distilled water. The medium was autoclave and allowed to cool, a single colony of DH5α Cells was inoculated into 10ml of LB medium and grown for 16 hours (overnight) at 37^0 C to get primary culture. 500µl of the primary culture was inoculated into 100 ml of fresh LB medium and grown for 2-3 hours at 37^0 C with vigorous shaking, when the O.D. of the culture reached 0.4, it was transferred to a 50ml falcon tube (half volume in each tube). The culture was cooled at 4^0 C on ice for about 30 minutes, the cells were recovered by centrifugation at 4500 rpm for 10 minutes at 4^0 C. The supernatant was discarded and the pellet was re-suspended in 20ml of 0.1M $CaCl_2$ and stored on ice for 30 minutes, it was then centrifuge at 4500 rpm for 10 minutes at 4^0 C, the supernatant was discarded and the pellet was re-suspended in 20ml 0.1M of $CaCl_2$ and stored on ice for 30 minutes. Centrifuge at 4500 rpm for 10 minutes and the supernatant were discarded, to the pellet 1ml of 0.1M $CaCl_2$ containing 10% glycerol was added and mixed. In 1.5ml Eppendorf, 100 microliters of the re-suspended cells were aliquot and stored at -80^0 C. (the cells were used directly for transformation). [25]

2.2 Transformation with HBx vector by heat-shock method

From 35ng of the HBx plasmid DNA, 10µl was taken and added to 100 microliters of DH5α competent Cells in two different tubes. They were allowed to incubate on ice for 30 minutes, the tubes were then transferred to 42^0 C water bath for 90 seconds so as to give bacterial cells heat shock. It was kept on ice for 5 minutes, 1ml of fresh LB media was added to both the two tubes and kept in shacking incubator at 37^0 C for 45-60 minutes. The pellet is recovered by centrifugation at 1000 rpm for 5 minutes, the pelleted cells were then suspended in 100µl of the media. The total volume was plated on agar plate that contained antibiotic (ampicillin (100µg/ml) for HBx, inoculation loop was used to spread the cells all over the plates. The plate was kept in incubator at 37^0 C overnight (for 16 hours).

2.3 Plasmid isolation of HBx by midi-preparation using alkaline lysis method

10ml of LB media containing antibiotics ampicillin (100µg/ml) for HBx was inoculated with a single colony of transformed bacteria. Incubate the culture overnight at 37^0 C with vigorous shacking to get the primary culture, 500µl of primary culture was added to 100ml of fresh LB-media containing respective antibiotic. Transfer the culture into 50ml tube and recover the bacteria pellet by centrifugation at 7000 rpm for 10 minutes at 4^0 C, remove the medium by gentle aspiration, leaving the bacterial pellet as dry as possible. Re-suspend the bacterial pellet in 200 microliter of ice-cold alkaline lysis solution I and add 4 microliters of RNase- A (1mg/ml) by vigorous vortexing and transfer the suspension to micro tube. Add 400 microliter of freshly prepared alkaline lysis solution II to each of the bacterial suspension, close the tube tightly, and mix the contents by inverting the tubes rapidly five times. Do not vortex. Store the tube on ice for 5-10 minutes, add 300 microliter

of ice-cold alkaline lysis solution III. Close the tubes and disperse alkaline lysis solution III through the viscous bacterial lysate by inverting the tubes several times. Store the tubes on ice for 3-5 minutes, centrifuge the bacterial lysate at maximum speed for 5 minutes at 4^0 C in a microfuge. Transfer 600 microliter of the supernatant to a fresh tube, add an equal volume of phenol: chloroform. Mix the organic and aqueous phase by vortexing and then centrifuge the emulsion at maximum speed for 10 minutes at 4^0 C in a microfuge, precipitate nucleic acid from the supernatant by adding 600 microliter of isopropanol at room temperature. Mix the solution by vortexing and then allow the mixture to stand for 10 minutes at room temperature, collect the precipitate nucleic acid by centrifugation at maximum speed at room temperature in a microfuge. Remove the supernatant by gentle aspiration, stand the tube in an inverted position on paper towel to allow all of the fluid to drain away. Remove any drops of fluid adhering to the walls of the tube. Add 1ml of 70% ethanol to the pellet and recover the DNA by centrifugation at maximum speed for 5 minutes at room temperature in a microfuge. Again remove all the supernatant by gentle aspiration, remove any beads of ethanol that form on the sides of the tube. Store the open tube at room temperature until the ethanol was evaporated and no fluid is visible in the tube (2-5 minutes), dissolve the nucleic acids in 100 microliters of TE (pH 8.0). Vortex the solution gently for few seconds, the DNA was stored at -20^0 C. [25]

2.4 Agarose Gel electrophoresis

Seal the edges of a clean, dry glass plate with tape to form a mold on horizontal section of the bench. Prepare sufficient electrophoresis buffer (1X TAE) to fill the electrophoresis tank and to cast the gel. Prepare a solution of agarose in electrophoresis buffer at a concentration for separating the particular size fragments expected in the DNA samples, 1% agarose of 40ml distilled water was prepared (i.e. 0.4g agarose was dissolved in 40ml distilled water). The flask containing agarose was boiled in microwave until the agarose dissolved, when the molten gel has cooled down, add ethidium bromide (EtBr) to a final concentration of 0.4ug/ml. Mix the gel solution thoroughly by gentle swirling, while the agarose solution is cooled, choose an appropriate comb for forming the sample slots in the gel. Position the comb 0.5 – 1.0 mm above the plate so that a complete well is formed when the agarose is added to the mold, pour the warm agarose solution into the mold. Allow the gel to set or polymerase completely (30-45 minutes at room temperature), then pour a small amount of electrophoresis buffer on the top of the gel, and carefully remove the comb. Pour off electrophoresis buffer, and carefully remove the tape. Mount the gel in electrophoresis tank, add enough electrophoresis buffers to cover the gel to a depth of approximately 1mm, mix the sample of the DNA with 2ul of 6x DNA loading buffer (loading dye). Slowly load the sample mixture into the slots of the submerged gel using a disposable micropipette, load sample size standards into slots on both the right and left sides of the gel. Close the lid of the gel tank, and attach the electrical leads so that the DNA will migrate toward the positive anode (red lead), apply voltage of 1-5v/cm (measured

as the distance between the positive and the negative electrodes). If the leads have been attached correctly, bubbles should be generated at the node and the cathode (due to electrolysis). When the DNA samples or dyes have migrated a sufficient distance through the gel, turn off the electric current and remove the leads and lid from the gel tank. [25]

2.5 Cell Culture

HepG2 (liver hepatocellular carcinoma) cell line were cultured in a T-25 culture flask. The medium was discarded and cells were rinsed with 1x PBS (phosphate buffered saline), the cells were trypsinized by adding trypsin to the culture flask (trypsin an enzyme to detach adherent cells from the surface of the cell vessels). The culture flask was kept at 37^0 C in CO_2 incubator for 1-2 minutes, the flask was taken from the incubator and inverted 2-3 times. As the cells detach, neutralize the trypsin by adding 4ml of DMEM complete media and re-suspend the media by means of pipetting. Transfer the media to 15ml tube and cover the top opening with Para film. Centrifuge the media at 1000 rpm for 5 minutes at 25^0 C, then discard the media and re-suspend the cell pellet in 4ml complete media using a pipette. Transfer the cell suspension to a fresh T-25 flask, finally the culture flask was kept in CO_2 incubator at 37^0 C overnight (after 8 hours the cells will attached to the surface of the media).

2.6 Transient Transfection of HepG2 cells with HBx

15µl of the HBx plasmid DNA (2µg/µl) was aliquot into fresh 1.5ml Eppendorf. 36µl of polyethylenimine (PEI 1mg/ml) was added to the same Eppendorf, 1149µl of DMEM incomplete media was also added to the tube and re-suspended. The tube was kept at room temperature for 30 minutes inside laminar flow, the flask with six wells incubated overnight was taken out from CO_2 incubator and the medium was discarded. 200µl of the incubated media containing PEI: DNA complex was aliquot into all the six wells of the culture flask through pipetting drop by drop in each well. 2ml of the complete DMEM media was also added to each of the six wells of the culture flask. The culture flask was kept in CO_2 incubator at 37^0 C for 48 hours and cells lysate was collected for SDS-PAGE and western blotting

Sodium dodecyl sulphate-polyacrylamide denaturing gel and sample preparation

2.7 Sample preparation

The transient transfected medium incubated at 37^0 C in CO_2 was taken out, 1ml of the medium was aliquot into 1.5ml fresh Eppendorf. It was centrifuge at 1000 rpm for 2 minutes. The supernatant was discarded, the rest of the medium left in each of the six wells of the culture flask were also added again into an appropriate Eppendorf in an orderly manner. The tubes were also centrifuge again at 1000 rpm for 2 minutes, 100µl of 1X RIPA lysis buffer containing 1µl of 100X protease inhibitor cocktail (PIC) was added to each of the six Eppendorf tubes. It was vortex and kept in -20^0 C. The tubes were transferred to ice in an inverted position, liquid nitrogen was sprayed all over the six Eppendorf tubes in order to increases the lysis of the cells. The tubes were centrifuge at 10,000

rpm for 30 minutes at 4^0 C, 20µl of each sample plus 5µl of 4x protein loading buffer was aliquot into fresh Eppendorf tubes. The tubes were heated at 95^0 C for 5 minutes in a water bath, the tubes were given short spin. [25]

2.7. 1 Pouring of SDS – PAGE Gels

Assemble the glass plates according to manufacturer's instructions. Determine the volume of the gel mold, prepare an appropriate volume of solution containing the desired concentration of acrylamide / bis-acrylamide for 12% resolving gel. Mix the component in an orderly manner, rapid polymerization will begin as soon as the TEMED has been added. Without delay, swirl the mixture rapidly and proceed to the next step, pour the acrylamide solution into the gap between the glass plates of the electrophoresis apparatus, leaving sufficient space for the stacking gel (length of the teeth of the comb plus 1cm). After polymerization is complete (30 minutes), pour off the overlay and wash the top of the gel several times with deionized H_2O to remove un-polymerized acrylamide, drain as much fluid as possible from the top of the gel, remove any remaining H_2O with the edges of a paper towel. In a disposable plastic tube, prepare the appropriate volume of the stacking gel mixture; polymerization will begin as soon as the TEMED has been added. Without any delay, swirl the mixture rapidly and proceed to the next step. Pour or pipette the stacking gel mix to fill the space above the resolving gel. Immediately insert a clean comb into the stacking gel solution, taking care to avoid trapping air bubbles. Add more stacking gel solution as needed to fill the space of the comb completely. Place the gel in a vertical position at room temperature.

2.7.2 Running the SDS-PAGE Gel

A**s** the stacking gel is polymerizing, add an appropriate volume of SDS gel-loading buffer. After polymerization is complete (30 minutes), carefully remove the comb. Use a squirt bottle to wash the wells immediately with deionized H_2O to remove un-polymerize acrylamide, 20µl of each samples plus 4µl of 4X loading dye were loaded along with the 3µl pre-stained protein marker. Attached the electrophoresis apparatus to an electric power supply (the red positive electrode should be connected to the bottom buffer reservoir). Apply a voltage of 120V, and run the gel until the bromophenol blue reaches the bottom of the resolving gel. Remove the gel sandwich from the apparatus using an opening key and use one part of the gel for staining and the other for transfer. [25]

2.8 Checking the protein expression of transfected cells with plasmid HBx by western blotting

Firstly, filled the tray with a loading buffer 1x Transfer buffer, containing 20% methanol. Remove the gel from the cassette using an opening key. Collaborate the tray in the Transfer buffer with tray for 15 minutes, suck the fiber pad in Transfer buffer. Place the gel holder cassette in the container with the black site down and white up. Lay one pad at the top of the gel cassette holder and put a blotting paper, be careful to avoid air bubbles. Place the gel carefully on the blotting paper and ensure that there are no air bubbles. Put the nitrocellulose membrane at the top of the gel, wait it before keeping in the top of the gel. Using the ruler remove any air bubbles between the membrane

and the gel, place another blotting paper at the top of the nitrocellulose membrane and place another fiber pad at the top of the blotting paper. Close the gel cassette holder by attaching black and white sides and clip it. Place the gel holder into the inner mage of the electrophoresis tank, make sure that the black side is next to the black side of the inner mag. Add a frozen cold unit, fill the gel with Transfer buffer up to the mark indicated. Apply the electric current by connecting red to red and black to black, set the voltage to 100v 0r 350 mill ampere. When the run is finish turn off the gel and remove the inner gel cassette and place in a container with Transfer buffer and place the black side in the buffer. Starting with the first fiber pad remove each layer until you reach the nitrocellulose membrane, as the membrane protein has completely transferred to both side of the membrane and no more protein in the gel. To the membrane ponceauos stains was added to check the quality of the transfer. The membrane was then washed with PBST and blocked in 5% BSA prepared in PBST. The membrane is kept for 1hour in 5% BSA on shaker. Pour off the blocking solution and add 10ml of primary antibody (Anti-GAPDH1:10,000) Dilution and incubate for overnight in a shaker. Pour off the primary antibody and rinse the membrane with the wash PBST. Add 20ml of the PBST and let it wash for 10 minutes. Discard the PBST and add fresh 20ml PBST and perform the above 2 more times. Discard PBST and add 10ml of secondary antibody (Anti-mouse 1:10,000) and kept it on a shaker for 2 hours at room temperature. Pour off secondary antibody and rinse the membrane with the PBST, and add 20ml PBST and let it wash for 10 minutes. Discard the PBST and proceed for immuno-blotting.

2.8.1 Development of X-ray film by (enhanced chemiluminescence)

10ml 0.1M Tris-Cl pH 8.5, 50µl luminol, 22µl PCA and 5µl H_2O_2 was added into a fresh falcon tube. 3 containers were set; first with developer solution, second with water and third with fixer solution. Take the incubated nitrocellulose membrane with secondary antibody and discard the PBST, pour the solution of Tris-Cl, luminol, PCA and H_2O_2 into the membrane and leave it for some time. Cut the X-ray film proportional to the size of the membrane and kept the gel in between the plastic sheet inside X-ray clip. Put the X-ray film immediately at the position of the gel and close the clip and tied it for some time. Transfer the x-ray film to the first container with developer solution and dip it for 1-2 minutes, and then transfer it to the next container with water and dip it. Finally, transfer it to the next container with fixer solution and dip it as well. Repeat the above process for about 2-3 times, Varying the exposure time of X-ray film to the membrane. Keep the X-ray film in an inverted position in order to drain properly, Remove the membrane from the X-ray clip and pour PBST to it. [25]

3. RESULTS AND DISCUSSION

3.1.1. Transformation of DH5α with HBx plasmid vector

The transformation of the *E. coli* (DH5α) bacterial cells was done with the HBx plasmid DNA which possess ampicillin resistant gene, as described in material and method by heat-shock method. The plasmid DNA is available in the laboratory for experimental purposes, the transformed cells were grown on Luria Agar plates (LA) containing ampicillin (100µg/ml) antibiotic. The transformation of bacterial cells was carried out using 10µl (35ng/µl) HBx. Fine colonies of *E. coli* bacterial cells were observed on LA plate containing ampicillin (antibiotic) which indicated the success and free contamination of the transformation

Figure 3.2.1: Showing bacterial colonies of DH5α cells transformed with 35ng/µl of HBx plasmid vector, containing antibiotic (100µg/ml) ampicillin.

3. 2.2 Midi-preparation of HBx

A single colony from transformed plates was picked up and grown overnight at 37^0 C in 200ml Luria broth (LB), in a shaking incubator containing ampicillin (antibiotic) for HBx (100µg/ml) antibiotic. The grown cells were harvested for HBx plasmid isolation. Midi-preparation was done by the method described in material and method. The plasmid isolated as indicated in material and method were run in 1% agarose. 2µl of the plasmids was loaded plus 2µl of 6X DNA loading dye along with marker (1Kb DNA ladder). The Plasmid DNA has got a good conformation in aqueous medium, and the band is observed after run on agarose gel.

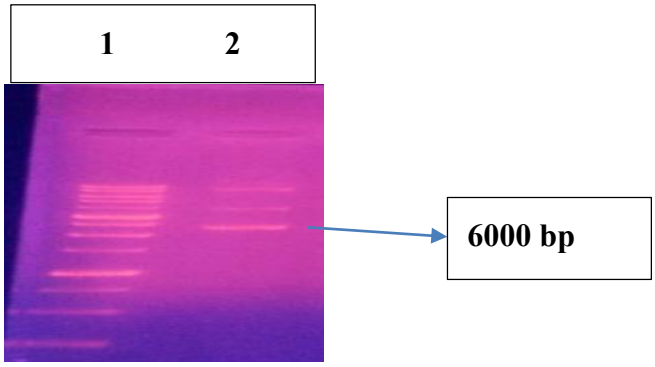

Figure 3.2.2: plasmids HBx isolated by midi-preparation.

3.2.3 Transient Transfection of HepG2 cells with HBx

Transient transfection was performed with HepG2 cell culture after the cells have attached to the surface of the wells, 200µl of incomplete media and 15µl of the plasmid HBx (2µg/µl) was aliquot into fresh 1.5ml Eppendorf. Polyethylenimine (PEI (1mg/ml) -(21µl) was added to the same tube as explained in the protocol of transient transfection in material and method. The culture plate was then kept in CO_2 incubator at 37^0C for 48 hours. The cells lysate was collected and prepared for SDS-PAGE and western blotting as described in material and method. Figure 3.2.3 below illustrates transient transfection of HBx after visualized under fluorescent microscope. As the excitation of HBx is at 244 nm a blue light was used for causing the excitation of HBx protein. A green fluorescence coloured was observed corresponding to the emission of HBx at 257 nm, the green fluorescence indicated that HBx protein is showing its expression on transiently transfected HepG2 cells.

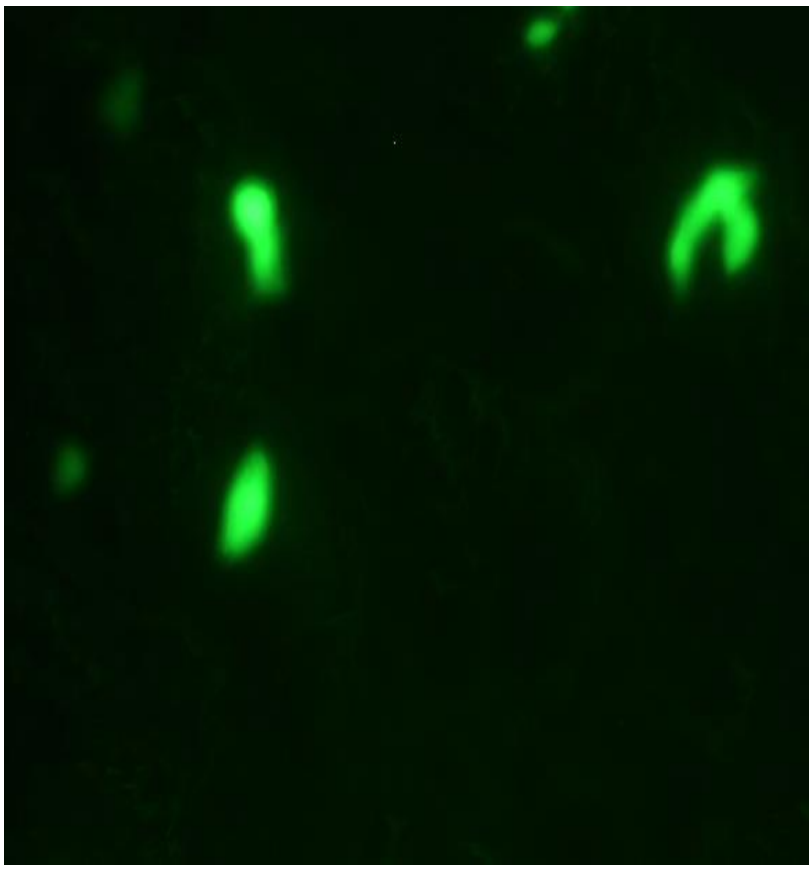

Figure 3.2.3: Showing fluorescent microscopy image of HBx transiently transfected in HepG2 Cell line.

3.2.4 Sodium dodecyl sulphate-polyacrylamide denaturing gel and sample preparation

The samples are prepared from the transiently transfected HepG2 culture incubated at 37^0C in CO_2 incubator, 1µl of 100X PIC and100µl of 1X RIPA Lysis buffer was added to each wells. The cells

were scrapped and collected in a microfuge, it was centrifuge and 4X loading dye was added to each tube as shown in the protocol of SDS-PAGE in material and method. The samples were heated at 95^0C for 5 minutes in a water bath, and then run on 12% SDS-PAGE by loading 20µl of the sample plus 4µl of 4X loading dye along with 3µl marker (pre-stained protein marker). After the run has finished the gel was transferred to nitrocellulose membrane for immunoblotting.

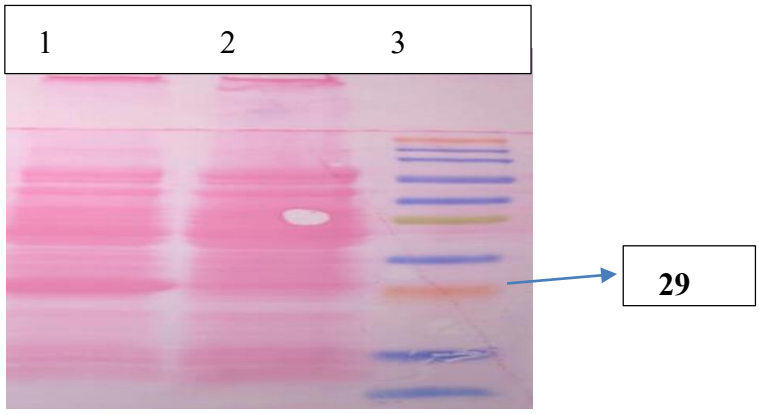

Figure 3.2.4: Ponceau S Stained nitrocellulose membrane

3.2.5 Checking the expression of HBx in transiently transfected cells by western blotting

The protein of SDS-PAGE gel was transferred into nitrocellulose membrane as mentioned in material and method, Ponceau S stain is used to check the efficiency of the transfer as shown in Figure 3.2.4 above. The membrane is washed with PBST (Phosphate buffered Saline) and blocked using 5% BSA (Bovine serum albumen, American origin) prepared in PBST (since the membrane has higher affinity for binding). The membrane is kept overnight in a shaker at room temperature incubated with primary antibodies dilution 1:10,000 (anti- HBx and anti- GAPDH), and washed with PSBT with 10 minute intervals, it's also incubated with secondary antibodies (anti-mouse) dilution 1: 10,000 as shown in the protocol of western blotting in material and method section. The membrane is taken to dark room for development of X-ray film **(**enhanced chemiluminescence) where it is soaked in a solution of 10ml 0.1M Tris-Cl (pH 8.5), 50µl luminol, 22µl PCA and 5µl H_2O_2. Finally, the membrane is covered with X-ray film and placed in X-ray cassette. The X-ray film is then treated with developer solution, water and fixer. The bands appeared on the X-ray films are used for specific protein detection and analysis. The Figure 3.2.5 and 3.2.6 below illustrate the protein expression of HBx protein in the absence of HBx protein is taken as control and in the presence of HBx protein taken as control. GAPDH (Glyceraldehyde 3 phosphate dehydrogenase) was used as a loading control.

[A] Fig. 3.2.5: Western blotting:

1 2

[B]Fig 3.2.6 Western blotting:

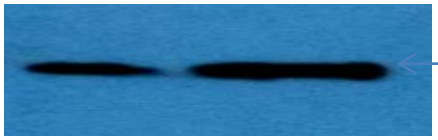

Figure 3.2.5 & 2.5.6: Immunoblotting of HBx

DISCUSSION

The HBx plasmid DNA isolated by mid-preparation using alkaline lysis method was analyzed using agarose gel electrophoresis, results observed from the gel showed that HBx DNA has got a good conformation up to 6000 base pair and can use for further research/investigation. The DNA was transiently transfected in HepG2 cell line; its protein expression had been observed under florescent microscope as the excitation of HBx is at 244 nm a blue light was used for causing the excitation of HBx protein. A green fluorescence colored was observed corresponding to the emission of HBx at 257nm, the green fluorescence indicated that HBx protein is showing its expression on transiently transfected HepG2 cells. The samples were collected and prepared for SDS-PAGE, the protein has been separated according to their molecular weight by SDS-PAGE and transferred into nitrocellulose membrane for immune-blotting. The efficiency of protein separation is checked with Ponceau S Stained prior to the transfer. The protein expression was verified analytically through western blotting or immunoblotting a technique that detect specific expression of protein. HBx protein bands has correspond to 40KDa and 33KDa in both absent and present of loading control, GAPHD was used as loading control. Therefore, from this study it can be postulated that HBx is responsible for protein expression of HBV and responsible for hepatocellular carcinoma (HCC) during hepatitis B virus infections in hepatocytes (liver cells). Studies of HBx deletion mutants have identified domain of HBx responsible for its function. The transactivation function of HBx resides between amino acids (aa) 52-148, whereas aa 1-50 encode a domain that can inhibit HBx activities [20, 31]. Further studies defined aa 120-140 as involved in signal transduction activities, and the carboxyl-terminal 20 aa as involved in HBx stability [22, 23]. HBx aa 54-70 were later shown to be essential to HBx localization in mitochondria, whereas aa 75-88 and 109-131 were shown to aid in the localization of HBx to mitochondria [24]. HBx aa 82-154 are sufficient to mediate bindings to proteasome subunits [34, 36], whereas p53 binding is mediated by aa 102-136 [27,32]. It is important to note, however, that without a defined 3D structure of HBx as a basis for studying mutant HBx proteins, it remains unclear where observed effects of the mutant HBx proteins that were

analysed reflected identification of regions of HBx, which are required for specific functions or were caused by disrupting the overall structure. An important goal in treatment for hepatitis B is to prevent the development of HCC for which there is presently no effective therapy. As the integration/ expression of the x-gene has been observed in majority of the HBV-related HCC [28, 29} an effective way to control this disease would be to interfere with HBx expression. Attempts have been made in the recent past to regulate the expression of HBx in cell culture and experimental animals using anti-HBx antibody, antisense oligonucleotide, ribozyme or even small inhibitory RNAs. According to a report from China. treatment of nude mice (having human HCC grafts) with anti-HBx antibody leads to significant tumor regression and prolonged survival [30]. Further, HCC patients treated with anti-HBx antibody also show reduction in tumor size and a decrease in the serum level of alpha fetoprotein [31]. The antisense phosphorothioate oligonucleotides directed against the initiation codon of the x-gene can prevent the development of pre-neoplastic lesions in a transgenic mouse model of HCC [32]. Some hammerhead ribozymes have also been designed that can inhibit the expression of functional HBx and show anti-replicative effect in cell culture [33]. This illustrates the potential of ribozymes in the treatment of chronic liver disease and HCC. Nowadays, RNA interference (RNAi) is being used as a powerful antiviral strategy to silence virus-specific genes. Expression of short hairpin RNAs directed against the x-gene appears to induce an RNAi.

4. CONCLUSION

In conclusion, HBx was found to be required for protein expression of HBV in HepG2 cells, it is also responsible for hepatocellular carcinoma (HCC) in hepatocytes during HBV infection. This finding ascertain that the effects of HBx on HBV replication and transcription vary depending on the experimental system. This discrepancy may be due to different cell genetic backgrounds and/or environments, but the exact reasons remain unclear. In order to get a more realistic understanding of the roles of HBx in HBV transcription and replication, it is necessary to perform investigations under physiological conditions. In a nutshell, HBx is responsible for HBV replication, cell circle regulation, hepatocellular carcinoma (HCC), signal transduction, apoptosis (cell death), cell transformation, therapeutic application, viral life cycle and DNA repair. Therefore, clear understanding of HBx protein expression and inhibition will serve as important tool for developing HBV therapy. In this study, the HBx plasmid DNA transiently transfected in HepG2 cell line showed its protein expression and verified analytically through western blotting or immune-blotting a technique that detects specific protein expression. HBx is responsible for HCC and protein expression of HBV in hepatocytes during infection. The results obtained from this research will be beneficial to researchers, academicians, scientist, physicians, pharmacists and various health regulatory agencies

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The author confirms that the data supporting the findings of this research are available within the article.

FUNDING

The research work was funded by: **Tertiary Education Trust Fund** (**TETfund**) Abuja, Nigeria.

ACKNOWLEDGEMENT

The author wish to thank **Tertiary Education Trust Fund** (**TETfund**) Abuja, Nigeria for support and funding of the research work. He also thanks the director Centre for dryland Agriculture, Bayero university, Kano and the coordinator molecular biology laboratory Aminu Kano teaching hospital for granting permission to carry out some experiments/research work. Special thanks goes to Rabiu Sabo, Dr. Ibrahim Babale Gashu'a (PhD), Sani Ibrahim and Abubakar Chiroma for their assistance throughout the research work.

CONFLICT OF INTEREST

Authors have no conflict of interest.

REFERENCES

1. Seeger C, Mason Mc. Hepatitis B virus biology-Microb Mol Biol: Rev. 1984; 64**:** 51-68.
2. Bergametti F, Prigent S, Luber B, Benoit A, Tiollais P, Sarasin A. et el. The proapoptotic effect of hepatitis B virus HBx protein correlates with its transactivation activity in stably transfer cell lines. Oncogene. 1999; 18**:**2860-2871.
3. Gavilanes F, Gonzales A, Peterson D. Structure of hepatitis B surface antigen: characterization of the lipid components and their association with the viral proteins. J Biol Chem. 1982; 257:7770–7777.
4. Gerlich W, Robinson WS. Hepatitis B virus contains protein attached to the 5′ end of its complete strand. Cell. 1980; 21:801–811.
5. Hatton T, Zhou S, Standring DN. RNA- and DNA-binding activities in hepatitis B virus capsid protein: a model for their roles in viral replication. J Virol. 1992; 66:5232–5241.
6. Nishioka K, Suzuki H, Mishiro S, Oda T. Viral Hepatitis and Liver Diseases. Toky: Spger Vlf. 1994; 21: 743-7.
7. Hu Z, Zhang Z, Kim JW, Huang Y, Liang TJ. Altered proteolysis and global gene expression in

hepatitis B Virus X transgenic mouse liver. J Virol. 2006; 80:1405–1413.

8. McMahon BJ, Alward WL, Hall DB, Heyward WL, Bender TR, Francis DP. Acute hepatitis B virus infection: relation of age to the clinical expression of disease and subsequent development of the carrier state. J Infect Dis. 1985; 151:599–603.
9. Barker LF, Murray R. Relationship of virus dose to incubation time of clinical hepatitis and time of appearance of hepatitis–associated antigen. Am J Med Sci. 1972; 263:27–33
10. Berk PD, Popper H. Fulminant hepatic failure. Am J Gastroenterology. 1978; 69:349–400.
11. Hoofnagle JH, Carithers RL, Shapiro C, Ascher N. Fulminant hepatic failure: summary of a workshop. Hptlogy. 1995; 21:240–252.
12. Mahon MC. The natural history of chronic hepatitis B virus infection. Hptlogy. 2009;49: S45–S55.
13. Belloni L, Pollicino T, NicolaF DE, Guerrieri F, Raffa G, Fanciulli M. et. el. Nuclear HBx binds the HBV minichromosome and modifies the epigenetic regulation og cccDNA, function. Proc Natl Acad Sci: 2009; 106**:**19975-19979.
14. Li T, Robert EI, Breugel PC, Strubin M, Zheng N. A promiscuous alpha-helical motif anchors viral hijackers and substrate receptors to the CUL4-DDB1 ubiquitin ligase machinery. Nat Struct Mol Biol ,2010;17: 105–111
15. Cortazar D, Kunz C, Saito Y, Steinacher R, Schar P. The enigmatic thymine DNA glycosylase. DNA Repair. Amst. 2007; 6: 489–504.
16. McMahon BJ, Alward WL, Hall DB, Heyward WL, Bender TR, Francis DP. Acute hepatitis B virus infection: relation of age to the clinical expression of disease and subsequent development of the carrier state. J Infect Di*,* 1985; 151:599–603.
17. Barker LF, Murray R. Relationship of virus dose to incubation time of clinical hepatitis and time of appearance of hepatitis–associated antigen. Am J Med Sci. 1972; 263:27–33
18. Berk PD, Popper H. Fulminant hepatic failure. Am J Gastroenterology, 1978; 69:349–400.
19. Hoofnagle JH, Carithers RL, Jr, Shapiro C, Ascher N. Fulminant hepatic failure: summary of a workshop. Hptlogy. 1995; 21:240–252.
20. Mahon MC. The natural history of chronic hepatitis B virus infection. Hptlogy. 2000;49: S45–S55.
21. Weissberg JI, Andres LL, Smith CI, Weick S, Nichols JE, Garcia G. Survival in chronic hepatitis B. An analysis of 379 patients. Ann Intern Med.1984; 101:613–616.
22. Milich D, Liang TJ. Exploring the biological basis of hepatitis B e antigen in hepatitis B virus infection. Hptlogy. 2003; 38:1075–1086.
23. Lok AS, Lai CL. Alpha-fetoprotein monitoring in Chinese patients with chronic hepatitis B virus infection: role in the early detection of hepatocellular carcinoma. Hptlogy.1989;9:110–115
24. Spangenberg HC, Thimme R, Blum HE. Serum markers of hepatocellular carcinoma. Semin

Liver Dis. 2006; 26:385–390.

25. Sambrook & Russell. Molecular cloning-A laboratory manual. New York; Cold spring laboratory press, 2001.
26. Lin Y, Nomura T, Murakami S. The transactivation and p53-interaction functions of HBx are mutually interfering but distinct. Cancer Res 1997; 57**:** 5137-42.
27. Andrisani OM. Deregulation of epigenetic mechanisms by the Hepatitis B Virus X protein in hepatocarcinogenesis. Viruses. 2013; 5**:** 858-872.
28. Barrett TE, Scharer OD, Savva R, Brown T, Jiricny J. Crystal structure of a thwarted mismatch glycosylase DNA repair complex. EMBO J. 1999;18: 6599–6609.
29. Barrett TE, Savva R, Panayotou G, Barlow T, Brown T. Crystal structure of a G: T/U mismatch-specific DNA. EMBO J. 1999;18: 6599–6609.
30. Barry M, Fruh K. Viral modulators of Cullin RING ubiquitin ligases: Culling the host defense. Sci STKE. 2006;335**:** 1-5.
31. Lok AS, Lai CL. Alpha-fetoprotein monitoring in Chinese patients with chronic hepatitis B virus infection: role in the early detection of hepatocellular carcinoma. Hptlogy. 1989; 9:110–115.
32. Blum HE, Zhang ZS, Galun E, Weizsacher F, Garner B, Liang et el. Hepatitis B Virus X protein is not central to the viral life cycle in vitro. J virol. 1992*;* 66**:** 1223-1227.
33. Bouchard MJ, Puro RJ, Wang L, Schneider RJ. Activation and inhibition of cellular calcium and tyrosine kinase signaling pathways identify targets of the HBx protein involved in hepatitis B virus replication. J Virol. 2003*;* 77**:** 7713-7719.
34. Benhenda S, Cougot D, Buendia MA, Neuveut C. Hepatitis B Virus X protein molecular functions and its role in virus life cycle and pethogenesis. Adv Cancer Res. 2009; 103: 75-109.
35. Hollinger FB, Liang TJ. Hepatitis B virus. PPD-PA: Lppcott-Rv Publishers; pp. 2001; 2971–3036.
36. Benkirane ML. Methyltransferase PRMTI is a binding partner of HBx and a negative regulator of hepatitis B virus transcription. J virol. 2013;87**:** 4360-4371.
37. Lok AS, Lai CL. Alpha-fetoprotein monitoring in Chinese patients with chronic hepatitis B virus infection: role in the early detection of hepatocellular carcinoma. Hptlogy. 1989; 9:110–115.