Evaluating the Role of Hepatitis B virus, HBx Chimeric Protein, in Hepatocellular Carcinoma

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Abstract— **Hepatocellular Carcinoma (HCC), the most common form of primary liver cancer, has been reportedly linked epidemiologically to the chronic infection of Hepatitis B Virus (HBV). During chronic infection, HBV is observed to be integrated into the host genome and HBx- host chimeric transcripts are often observed in HCC patients. In this study, we examined HepG2 cells stably expressing either vector only (VC), Wild-type HBx (WT) or HBx-host chimera (P1T). This study aims to investigate the effects of HBx-host chimerism on the progression of HCC by studying the hallmarks of cancer: resistance to apoptosis, sustained proliferation and transformation. Western blot analyses, Real Time RT-PCR and immunofluorescence assays were initially performed to ensure that the transfected proteins were adequately expressed by the stable cell lines. Cell proliferation was then observed through the cell counting assay and the cell confluency assay while its transformation ability was assessed using the soft agar assay. The apoptosis assay was done to investigate programmed cell death under the induction of chemotherapy drugs. P1T had a significantly higher level of cell proliferation compared to WT and VC. This correlated with the upregulation of PCNA, a known cell proliferation marker which is involved in the cell cycle. P1T was also observed to have a higher level of resistance to chemotherapy induced cell death compared to WT. Overall, this study showed that the HBx-host chimeric P1T protein enhances HepG2 cell proliferation and may promote HCC progression.**

Keywords- HBX-host chimeric protein, HBV, HCC, proliferation, apoptosis, transformation, IncuCyte

I. INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer, accounting for half a million deaths every year (Poustchi; et al, 2010). Its development has been linked epidemiologically to the chronic infection of Hepatitis B Virus (HBV) (Bisceglie, 2011). Recent studies show that HBV infections are implicated in more than 50% of HCC worldwide (Guerrieri; et al, 2013), with 350 million people infected with HBV at greater risk of HCC (Nguyen; et al, 2009).

HBV is a double-stranded DNA virus and its genome encodes seven proteins: preS1, preS2, HBsAg, HBeAg, polymerase, core protein, and X protein (HBx) (Homs; et al, 2011). The preferred region within the HBV genome involved in integration is at the 3′-end of HBx. The HBx–human chimeric transcripts can be expressed as chimeric proteins (Toh; et al, 2013). This project will focus on the HBx gene as it is often included in the HBV DNA that integrates into the human cellular DNA which is found to be functionally active in HCC (Kew, 2011).

HBx-host chimerism was detected in HCC patients via DNA sequencing (Toh; et al, 2013) and stable expression was achieved in the HepG2 cell line (ATCC, USA), obtained from liver tissue in HCC patients. HepG2 is an effective in vitro system for the investigation of the expression and replication of HBV (Sells; et al, 1987). We examined the HepG2 cells stably expressing either vector only (VC), Wild-type HBx (WT) or HBx-host chimera (P1T). The VC acts as a negative control for confounding factors; WT codes for the Wild-type HBx protein; P1T codes for the HBx-host chimeric protein in figure 1.
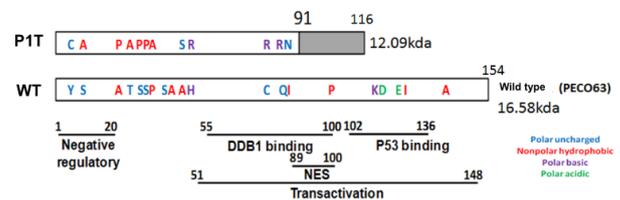

Figure 1: The HBx-host chimeric protein (P1T) and the Wild-type HBx (WT) protein examined, with functional domains labelled. The grey area represents the human region.

One of the major molecular mechanisms of hepatocarcinogenesis by HBV infection is the integration of the HBV DNA into the host genome (Hai; et al, 2014) which affects gene expression and results in the genetic alteration of the host genome (cis effect) or the HBV genome (trans effect). Furthermore, the transcription of HBx can affect the expression of growth control genes such as by inactivating the tumour suppressor p53, hence disrupting DNA damage checkpoints in the cell cycle, increasing cell proliferation (Farazi; et al, 2006).

The aim of this project is to investigate the effects of the HBx-host chimeric protein on hepatocarcinogenesis by studying the hallmarks of cancer: resistance to apoptosis, sustained proliferation, and transformation.

II. MATERIAL AND METHODS

A. Real Time Reverse-Transcriptase PCR

Using the QIAShredder (Qiagen, Germany), 500,000 cells of VC, WT and P1T were harvested and homogenised. The RNA was purified and measured using the RNeasy Mini Kit (Qiagen, Germany) and the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) respectively. Next, 500 ng of

RNA was diluted using 9 μl of water. Superscript II Reverse Transcriptase (Thermo Fisher Scientific, USA) was used to transcribe the RNA into cDNA. The primers used were GAPDH[1], WT[2] and P1T[3]. SYBR® Green (Thermo Fisher Scientific, USA) was used as a double-stranded DNA-specific dye. The data was then collected using the Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, USA).

B. Western Blot

HepG2 stable cells harvested in different conditions were used for protein extraction. Proteins were extracted with RIPA buffer (Thermo Fisher Scientific, USA) with protease inhibitor for 30 minutes at 4 °C, followed by centrifugation at 13,000 rpm for 30 minutes. Proteins were separated by Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and immunoblotting experiments were conducted using the 1: 8000 Anti-HBx rabbit polyclonal primary antibody (Abcam, UK); 1: 20,000 Anti-GAPDH rabbit polyclonal primary antibody (Merck Millipore, USA) and the 1: 2000 Proliferating Cell Nuclear Antigen (PCNA) mouse primary antibody (Research Biolabs, Singapore).

C. Immunofluorescence Assay

Cells were plated onto sterile coverslips in a 6-well plate at a density of 10,000 cells/cm2 in each well and incubated overnight, then fixed with 2% formaldehyde in Phosphate Buffered Saline (PBS) for 30 minutes in the dark at RT. The cover slips were then incubated with quenching solution (100 mM of Glycine in PBS) for 15 minutes and blocked with blocking solution (10% fetal calf serum in PBS) for 1 hour in the dark at RT. The cover slips were then incubated with 150 μl of 1:200 Anti-HBx rabbit polyclonal primary antibody and 1: 1000 Alexa Fluor 568 Phalloidin (Thermo Fisher Scientific, USA) for 1 hour in the dark at RT, then with 150 μl of 1:500 Alexa Fluor 488 Goat anti-Rabbit IgG (Thermo Fisher Scientific, USA) for 30 minutes in the dark at RT. 20 μl of mounting medium (Thermo Fisher Scientific, USA) was used to seal the coverslips to the slides. The immunofluorescence staining was then analysed by the Olympus FV1000 upright Microscope (Olympus, Japan) with the assistance of lab colleagues.

D. Cell Confluence Assay

The VC, WT and P1T cells were plated into a 96-well cell culture plate which was loaded into the IncuCyte ZOOM® System (Essen BioScience, USA). Three pictures of each well were taken every 3 hours for a period of 7 days. The cell number was estimated by the confluency of the cells.

E. Cell Proliferation Assay

Ten μl of cells was added under a coverslip on the haemocytometer and observed under the microscope using the 10x objective lens. The cells were counted manually by the average summation of the number of cells in three of the 16-grid squares. In a 6-well plate, 200,000 cells were plated in each well. Each of the 3 stable cell lines had 3 duplicates. One 6-well plate was counted each day, with the counting procedure repeated at the same time for 5 days.

F. Soft Agar Assay

To measure the proliferation rate of the three stable HepG2 cell lines, initial populations (32,000 and 64,000) of VC, WT and P1T were seeded into soft agar in a 6-well plate and incubated at 37 °C for 2 weeks. 2 ml of 0.005% crystal violet in methanol was added to each well, and the colonies were counted digitally using the OpenCFU software.

G. Apoptosis Assay

Camptothecin (CPT) (Abcam, UK) of 5 μM was added to 1x106 cells of VC, WT and P1T respectively. Equal volumes of Dimethyl sulfoxide (DMSO) (ATCC, USA) was added to the control wells. The cells were incubated for 16 hours at 37 °C, with 1x106 cells/ml resuspended in 1x Binding Buffer and 1x105 cells transferred to a 5 ml Fluorescence-activated cell sorting (FACS) tube. Five μl of PE Annexin V (BD Biosciences, USA) and 5 μl of 7-aminoactinomycin D (7-AAD) (BD Biosciences, USA) were added to the tube. After a 15 minute incubation at room temperature (RT) in the dark, 400 μl of 1x Binding Buffer (Thermo Fisher Scientific, USA) was added into each tube. The cells were analysed by the FACSCanto flow cytometer (BD Biosciences, USA).

III. RESULTS AND DISCUSSION

To quantitate the relative mRNA level of HBx, the Real Time RT-PCR was performed.
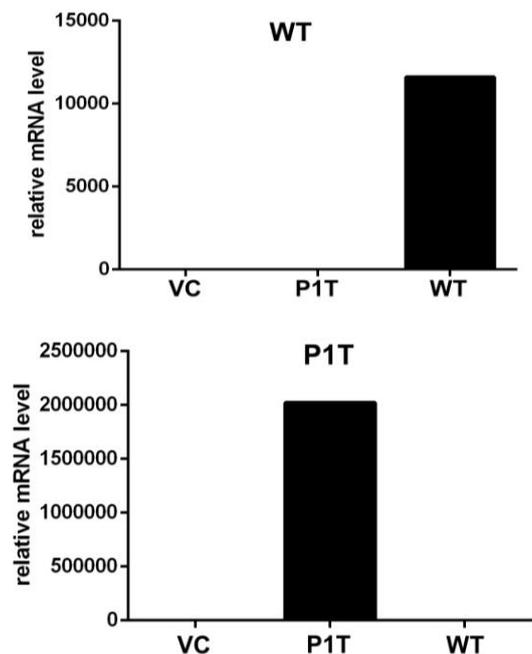

Figure 2: The relative mRNA level of VC, WT and P1T using WT HBx primer (top) and P1T HBx primer (bottom) in HepG2 stable cells

As observed from figure 2, the mRNA level of WT and P1T in control stables were normalized as 1, and the relative

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[1] forward - TGCACCACCAACTGCTTAGC; reverse - GGCATGGGACTGTGGTCATGAG
[2] forward - GGGACGTCCTTTGTTTACGTC; reverse - ACGGAGAAGGGGACGAGAGA
[3] forward - GCATGGAGACCACCGTGAAC; reverse - AAGGCTAGGTCTTGACCCCA

transcripts level of WT and P1T were 10,000 and 200,000 times more, compared to the control stables respectively. The mRNA level of P1T was 20 times more than the mRNA level of WT, which coincides with the protein level of WT and P1T in the presence of MG132.

To investigate the effect of HBx host chimeras on the progression of HCC, a western blot analysis was first carried out to observe the expression of the chimeric protein and the WT HBx. As HBx is relatively unstable and is present at low levels in the cell (Kim; et al, 2003), the proteasome inhibitor MG132 was added to reduce the degradation of HBx.
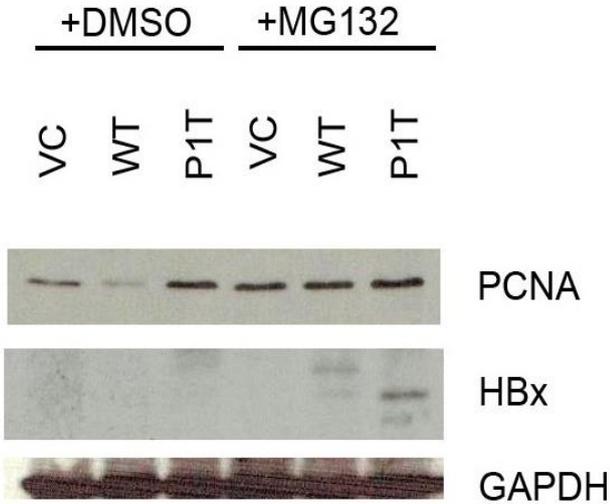

Figure 3: Western blot analysis of cell lysate from HepG2 stably expressing VC, WT or P1T, probing for PCNA (29 kDa), HBx protein (16.5 kDa) and GAPDH (37 kDa), with and without the MG132 drug

As observed in figure 3, the HBx protein was not detectable in HepG2 stable cells without the presence of MG132. WT (16.5 kDa) and P1T (12 kDa) HBx were observed when proteasome inhibitor MG132 was added. This indicates that the HBx protein has been integrated into the HepG2 cells and was expressed at an adequate level. The data also confirmed that WT and P1T HBx are both unstable and are degraded through a proteasome pathway. GAPDH was used for normalisation to show that identical amounts of samples were loaded. Therefore, the difference in experimental results would be solely due to the HBx-host chimeric protein. It is notable that in the presence of MG132, the protein level in P1T is slightly higher than in WT.

The immunofluorescence assay was carried out to observe the staining of HBx and the localisation of HBx in the cell.
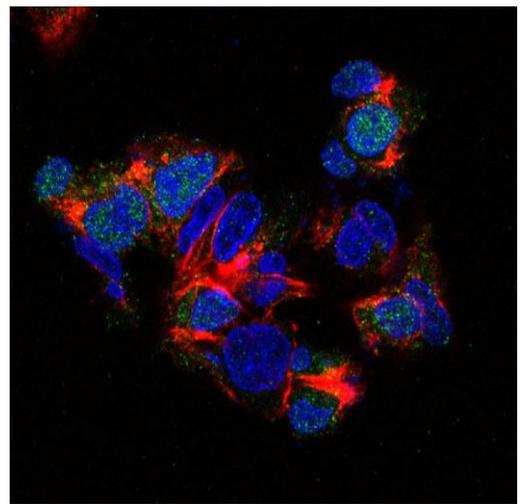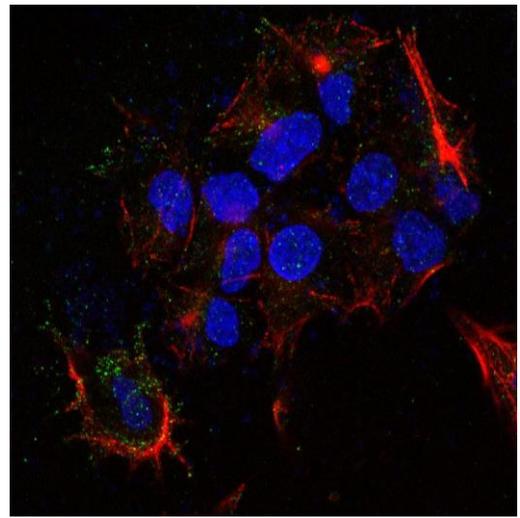

Figure 4: Confocal microscopy of HepG2 cells stably expressing VC (top) and WT (right), labelled with phalloidin (red), anti-HBx (green) and 4',6-diamidino-2-phenylindole (DAPI) (blue)

As seen in figure 4, the HBx antibody had a very high background in the immunofluorescence staining. There was a slightly stronger staining of HBx in WT compared to VC. The localisation of WT HBx was also observed to be in both the nucleus and cytoplasm. This may provide insights into the compartmentalisation of HBx for the understanding of its functions in the pathogenesis of HBx related diseases.

The cell confluence assay, cell counting proliferation assay and the soft agar assay was performed to investigate the rate of cell proliferation between the three stable cell lines.
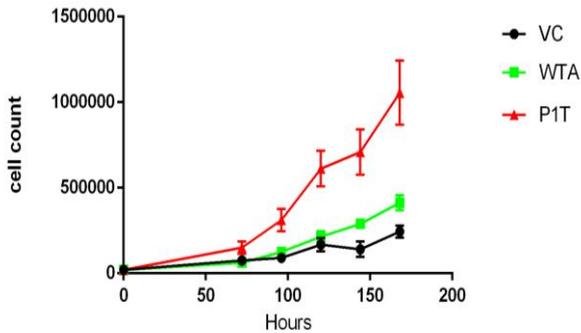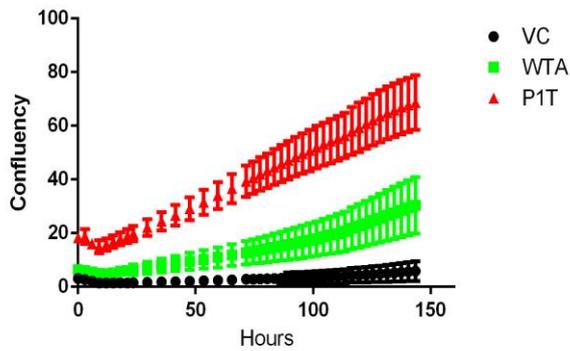
independent growth, which is a hallmark of carcinogenesis (Borowicz, *et al,* 2014).

PCNA is a specific marker for cell division (Strzalka; et al, 2011). As seen in figure 2, in the presence of MG132, comparable levels of PCNA were observed in the three cell lines, indicating that PCNA was stabilised. However, without MG132, P1T had a higher level of PCNA compared to VC and WT, indicating a higher level of cell proliferation due to the HBx-host chimeric protein. This is notable as in patients infected with HBV, the HBx-host chimeric protein may have disrupted the regulation of the cell cycle by upregulating the expression of PCNA which interacts with several eukaryotic cell cycle proteins such as p21 (Stoimenov; et al, 2009). This may be one of the mechanisms involved in the increase in cell proliferation due to the presence of the HBx-host chimeric protein.

To investigate the programmed cell death of VC, WT and P1T under drug induction, the Annexin V apoptosis assay was performed using CPT, which is commonly used in chemotherapy to induce apoptosis in cells (Zeng; et al, 2012).

Figure 5: Cell confluency assay over a period of 7 days (top) Cell proliferation counting assay over a period of 5 days with 3 days incubation beforehand (bottom) of VC, WT and P1T in HepG2 stable cells

As seen in figure 5, the cell confluency results showed that P1T had the highest percentage of confluence (68.7%), followed by WT (30.4%) and VC (5.87%). The cell proliferation counting results also showed that P1T had the highest cell count (1,056,111), followed by WT (413,333) and VC (243,750).
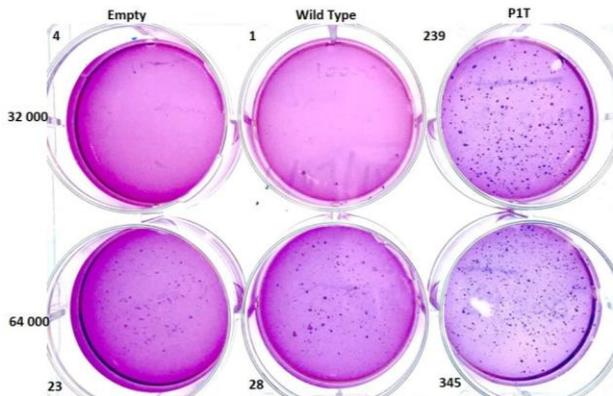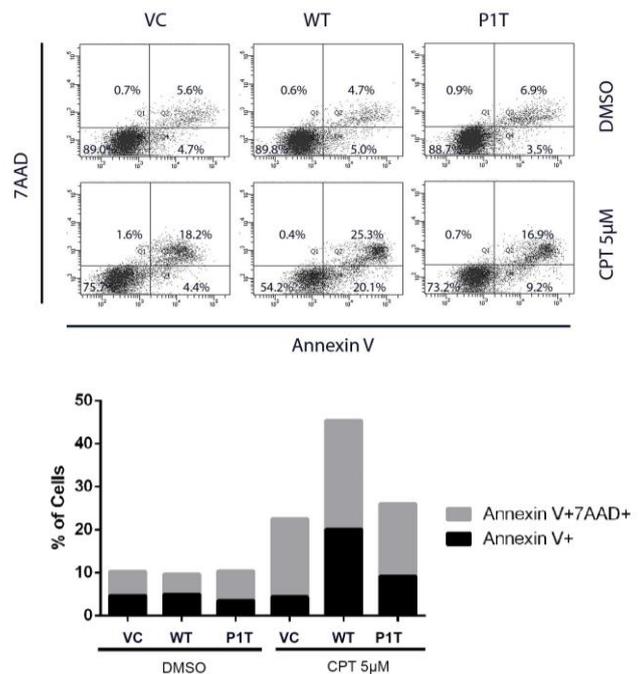

Figure 6: Soft agar assay experimental results of VC, WT and P1T in HepG2 stable cells at 32,000 and 64,000 starting cell numbers

A similar trend can be observed in figure 6 whereby P1T had more colonies (239) than WT (1) and VC (4) at the 32,000 starting cell number; P1T also had more colonies (345) than WT (28) and VC (23) at the 64,000 starting cell number. This indicates that the HBx-host chimeric protein may have disrupted cell cycle pathways, resulting in a higher cell proliferation rate compared to WT HBx, as well as anchorage-

Figure 7: (Top) Dot plots with Annexin V and 7AAD as the y and x axis respectively, with each HepG2 cell (dot) positioned in its respective quadrants (Q) of 7 AAD positive (Q1), Annexin V positive (Q3), or both Annexin V and 7 AAD positive (Q2). (Bottom) Chart depicting the percentages of HepG2 cells with and without the presence of CPT.

Annexin V identifies apoptotic cells by detecting phosphatidylserine at the outer plasma membrane. 7 AAD identifies cells with low membrane integrity as those undergoing the late apoptotic stage. As observed in figure 7, P1T has less apoptotic cells (26.1%) than WT (45.4%), but more than VC (22.6%). This indicates that even though cells infected with HBV tend to undergo apoptosis more than VC cells, the HBx-host chimeric protein increases the resistance to apoptosis, which is a hallmark of cancer. This may reduce the effectiveness of chemotherapy drugs targeted at cancer cells.

IV. Conclusion

The HBx-host chimeric protein may disrupt important cellular processes, leading to genetic instability and the progression of HCC. From our findings, the HepG2 P1T chimeric stables have a significantly higher level of cell proliferation compared to WT. Furthermore, the P1T chimera is more resistant to chemotherapy induced cell death suggesting HBV chimerism may reduce the effectiveness of treatment drugs in the pathogenesis of cancer. Since HBx is known to be involved in mitosis and apoptosis pathways (Reuben, 2015), high throughput genomics and proteomics studies such as microarray, RNA-sequencing, and protein mass spectrometry are required to investigate the HBx-host chimeric pathway and binding partners. This can shed light on the exact functions and mechanisms between HBx-host chimerism and cancer progression.

References

[1] Bisceglie, A.M.D. (2009) Hepatitis B And Hepatocellular Carcinoma. *Hepatology*, S56–S60.

[2] Borowicz, S., Scoyk, M., Avasarala, S., Rathinam, M., Tauler, J., Bikkavilli, R., & Winn, R. (2014). The Soft Agar Colony Formation Assay. *Journal of Visualized Experiments*

[3] Cheng, Q.; Yuan, F.; Lu, F.; Zhang, B.; Chen, T.; Chen, X.; Cheng, Y.; Li, N.; Ma, L.; Tong, T. (2015). CSIG promotes hepatocellular carcinoma proliferation by activating c-MYC expression. *Oncotarget*, 4733-4744.

[4] Farazi, P. & DePinho, R. (2006). Hepatocellular Carcinoma Pathogenesis: From Genes To Environment. *Nature Reviews Cancer*, 674-687.

[5] Guerrieri, F.; Belloni, L.; Pediconi, N. & Levrero, M. (2013). Molecular Mechanisms of HBV-Associated Hepatocarcinogenesis. Semin Liver Dis Seminars in Liver Disease, 147-156.

[6] Guzińska-Ustymowicz K.; Pryczynicz, A.; Kemona, A.; Czyzewska, J. (2009) Correlation between proliferation markers: PCNA, Ki-67, MCM-2 and antiapoptotic protein Bcl-2 in colorectal cancer. *Anticancer Research*, vol. 29 no. 8, 3049-3052.

[7] Hai, H.; Tamori, A.; Kawada N. (2014) Role of hepatitis B virus DNA integration in human hepatocarcinogenesis. *World Journal of Gastroenterology WJG*, 6236-6236.

[8] Hanahan, D.; & Weinberg, R. (2011). Hallmarks Of Cancer: The Next Generation. *Cell*, 646-674.

[9] He, Y.; Yang, F.; Wang, F.; Song, S.; Li, D.; Guo, Y.; & Sun, S. (2007). The Upregulation of Expressed Proteins in HepG2 Cells Transfected by the Recombinant Plasmid-containing HBx Gene. Scand J Immunol Scandinavian Journal of Immunology, 249-256.

[10] Henkler, F.; King, I.; Hoare, J.; Mcgarvey, M.; Waseem, N.; Koshy, R. & Goldin, R. (2001). Intracellular localization of the hepatitis B virus HBx protein. *Journal of General Virology*, 871-882.

[11] Homs, M.; Buti, M.; Quer, J.; Jardi, R.; Schaper, M.; Tabernero, D.; Ortega, I.; Sanchez, A.; Esteban, R.; and Rodriguez-Frias, F. (2011). Ultra-deep pyrosequencing analysis of the hepatitis B virus preCore region and main catalytic motif of the viral polymerase in the same viral genome. *Nucleic Acids Research*, 8457-8471.

[12] Kew, M. (2011). Hepatitis B virus x protein in the pathogenesis of hepatitis B virus-induced hepatocellular carcinoma. *Journal of Gastroenterology and Hepatology*, 144-152.

[13] Kim, J.; Kang, S.; Kim, J.; & Ahn, B. (2003). Hepatitis B Virus Core Protein Stimulates the Proteasome-Mediated Degradation of Viral X Protein. *Journal of Virology*, 7166-7173.

[14] Kim, J.; Sohn, S.; Yen, T.; & Ahn, B. (2008). Ubiquitin-dependent and -independent proteasomal degradation of hepatitis B virus X protein. *Biochemical and Biophysical Research Communications*, 1036-1042.

[15] Nguyen, V.; Law, M.; & Dore, G. (n.d.). Hepatitis B-related Hepatocellular Carcinoma: Epidemiological Characteristics And Disease Burden. *Journal of Viral Hepatitis*, 453-463.

[16] Poustchi, H.; Sepanlou, S.G; Esmaili, S.; Mehrabi, N.; Ansarymoghadam, A. (2010). Hepatocellular Carcinoma in the World and the Middle East. *Middle East Journal of Digestive Diseases*, 31-41.

[17] Reuben, A. (2015). Hepatocellular carcinoma in adults and children, an issue of clinics in liver disease. S.l.: Elsevier.

[18] Sells, M.; Chen, M.; & Acs, G. (1987). Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. *Proceedings of the National Academy of Sciences*, 1005-1009.

[19] Stoimenov, I.. & Helleday, T. (2009). PCNA on the crossroad of cancer. *Biochemical Society Transactions*, 605-605.

[20] Strzalka, W. & Ziemienowicz, A. (2010). Proliferating cell nuclear antigen (PCNA): A key factor in DNA replication and cell cycle regulation. *Annals of Botany*, 1127-1140.

[21] Toh S.; Lee, C. (2008) Role of the Hepatitis B Virus in Hepatocellular Carcinoma. *Karger*, vol 1, 94-107.

[22] Zeng, C.; Zhang, X.; Lin, K.; Ye, H.; Feng, S.; Zhang, H. & Chen, Y. (2012). Camptothecin Induces Apoptosis in Cancer Cells via MicroRNA-125b-Mediated Mitochondrial Pathways. *Molecular Pharmacology*, 578-586.