creative commons
COMMONS DEED

저작자표시-비영리-변경금지 2.0 대한민국

이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.

변경금지. 귀하는 이 저작물을 개작, 변형 또는 가공할 수 없습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer

dCollection

Master's Thesis

Hepatitis B virus X gene differentially modulate the cell cycle and apoptotic proteins in normal liver and hepatoma cell lines.

Chang – Hee Yang

Department of Medicine

Graduate School

Cheju National University

February, 2012

간세포와 간암세포에서 Hepatitis B virus X 유전자에 의한 세포주기와 세포자멸사 단백질의 다른 조절에 관한 연구.

지도교수 조 문 제

양 창 희

이 논문을 의학 석사학위 논문으로 제출함

2012 년 2 월

양창희의 의학 석사학위 논문을 인준함

심사위원장 박 덕 배 印

위 원 조 익 훈 印

위 원 조 문 제 印

제주대학교 대학원

2012 년 2월

dCollection @ jeju

Hepatitis B virus X gene differentially modulate the cell cycle and apoptotic proteins in normal liver and hepatoma cell lines.

Chang Hee Yang

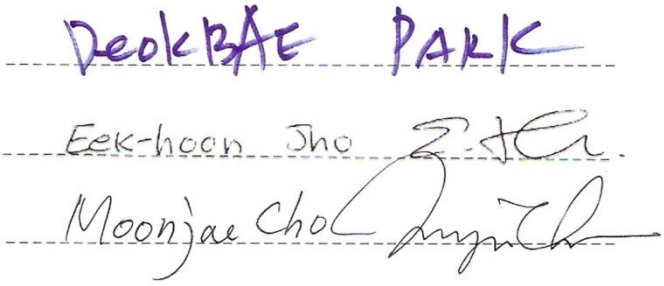
(Supervised by Professor **Moon jae Cho**)

A thesis submitted in partial fulfillment of the requirement for the degree

of Master of Science in Medicine

2011 . 12

This thesis has been examined and approved



Department of Medicine

Graduate School

Jeju National University

February,2012

dCollection @ jeju

JEJU NATIONAL UNIVERSITY 1952

CONTENTS

dCollection @ jeju

dCollection @ jeju

Collection @ jeju

LIST OF FIGURES

dCollection @ jeju

JEJU NATIONAL UNIVERSITY 1952
JEJU 1952
제주대학교

PART I

Hepatitis B virus X gene differentially modulate the cell cycle and apoptotic proteins in normal liver and hepatoma cell lines.

dCollection @ jeju

JEJU NATIONAL UNIVERSITY 1952

1. ABSTRACT

HBx is essential for early virus infection, HBV genome replication, HBV associated liver dieses and development of hepatocellular carcinoma(HCC). However, a exact role of HBx is still controversy. In this study, we focus and studied controversial role of HBx at the regulation of cell cycle and apoptosis in normal liver and hepatoma cell lines. We established a Huh7-X and Chang-X cell line that constitutively expresses HBx mRNA and protein. HBx proteins showed some difference in cell cycle regulation and expression of p27 and TGF-β between each cell lines. In addition, HBx proteins dramatically increased expression of Bcl-2 and reduced Cleaved PARP protein in Chang-X cells and inhibited the apoptosis in unfavorable condition such as serum starvation in Chang and Huh7 cells. Our findings demonstrate that HBx does a different role on to control of cell cycle progression and apoptosis in hepatoma cell and normal liver cell lines and provide some clues at the controversial issue of intracellular roles of HBx.

***Key words*:** HBx , HBV, p27 , cell cycle, apoptosis, TGF-β

dCollection @ jeju

2. INTRODUCTION

Hepatitis B virus (HBV) is one of the major risk factors for the hepatocellular carcinoma (HCC) and HCC-associated disease including liver cirrhosis (LC), acute hepatitis B (AHB) and chronic hepatitis B (CHB) [1]. Approximately 2billion people infected with the HBV in the world and above 400million people are chronically infected patients.

The HBV contains a 3.2 Kb length relaxed circular double stranded DNA genome and it contains four overlapping open reading frames (ORF) including surface gene (preS1/S2/S), core gene(pre-C/C), polymerase gene(P) and X gene[2]. The hepatitis B virus x (HBx) protein is a 17kDa nonstructural protein consist of 154 amino acid and encoded X gene.

HBx is essential for the various stage of virus cycle such as early virus infection, HBV genome replication, HBV associated diseases and development of hepatocellular carcinoma (HCC) [3, 4]. The expression of HBx affects a proliferation, differentiation, matastasis, and apoptosis in cancer cells through various signaling pathways. [5-9]. However, reported effects of HBx expression were not consistent. HBx promotes the cell cycle progression via down-regulation of cell cycle suppressive proteins such as p21 and p27 and up-regulation of cell cycle progressive factor such as Cyclin D, E, PCNA in Huh7, Chang and HepG2 cells [14, 15, 16]. However, HBx down-regulate the expression of Cyclin D1, E, A, B1, CDK 2, 4

Collection @ jeju

and PCNA and induce G1 phase arrest and repressed cell growth via GSK-3B/B-Catenin cascade in HBx stable expressed normal liver Chang cell line. Futhermore, when the Chang-HBx cells were injected in nude mouse, tumorigenesis was suppressed compared with Chang cells [10-13]. HBx is involved in down-regulation of apoptosis through up-regulation of anti-apoptotic proteins such as bcl2 and survivin proteins [2, 14-19]. In other study, HBx is reported to induce apoptosis through an increase of Bax, Bad expression and activation of Caspase8, Caspase3 activation [10-13].

TGF-β signaling related to both tumor-suppression and oncogenesis. TGF-β type 1 receptor (TBR1) activated by TGF-β and Smad3 phoshorylated in COOH-terminal (pSmad3C) domain, which transmits a tumor-suppressive TGF-β signal through up-regulation of p21(waf1). However, in hyperactive Ras-expressing transformed epithelial cells, TGF-β induced Smad3C phosphorylation is interrupted but the JNK pathway is stimulated and it is leading to phoshorylation on the linker region of Smad3. Activation of JNK-dependent pSmad3L pathway increases the transcriptional activity of c-Myc and promotes TGF-β induced oncogenic pathway. This pathway has been reported to induce the development of cancers in liver and colon [20-22]. HBx also preferentially activates JNK/pSmad3L/c-Myc oncogenic pathway over the TGF-β-mediated tumor-suppressive pSmad3C pathway in Early Chronic Hepatitis B [23].

Collection @ jeju

HBx is believed to influence the apoptosis, cell cycle and cell growth, however, an exact role of HBx is still unclear. In the present study, we focus and studied controversial roles of HBx on the regulation of cell cycle and apoptosis in normal liver and hepatoma cell lines.

dCollection @ jeju

3. MATERIALS AND METHODS

3.1. Cell culture and transfection

Huh-7(human hepatoma cell line) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum(FBS)(GIBCO inc, Grand Island, NY, USA). Chang(human normal liver cell line) cells were cultured RPMI-1640 medium with 10% FBS. Huh-7 cells and Chang cells were transfected with pEGFP-HBx plasmid and pCDNA3-HBx plasmid. For transfection, Lipofectamine 2000 (Invitrogen,Carlsbad,CA,USA) is used accoding to manufacturer's protocols. For stable transfection, 24hr after transfection, transfected cells were selected by G418 (500 ug ml^{-1}, AG Scientific, CA, USA) containing medium. The individual G418-resistant clones were selected and analyzed by PCR and westhern blot.

3.2. Plasmids

The HBx genes cloned into pGEM was donated by Dr. Song Byoung-Cheol (Cheju University , Cheju, Korea). A recombinant plasmid was made by HBx into the pEGFP-N1 or pCDNA3 plasmid vector. The insert was amplified by PCR using a pGEM template containing HBx with addition of XhoI and EcoRI restriction sites to primers matching those

Collection @ jeju

found in the pEGFP-N1 vector and pCDNA3 vector. The following primers(Integrated DNA Technologies) were used: HBx GFP-XhoI Forward 5'-ATCCTCGAGATGGCTGCTCGGGTGTGC-3' , HBx GFP-EcoRI Reverse 5'-GAT GAATTCCGGCAGAGGTGAAAAAGTTGC-3' and HBx-XhoI Forward 5'-ATACTCGAGATGGCTGCTCGGGTGTGC-3', HBx-EcoRI Reverse 5'-GCAGAATTCGGCAGAGGTGAAAAAGTTGC-3'. Restriction digest with XhoI and EcoRI-HF enzymes(New England Biolabs,Ipswich,MA,USA) was performed for both the insert and vector, followed by ligation with T4 DNA ligase(Promega, Madison, WI, USA).

3.3. Western blots and antibodies

Cells transfected with indicated plasmids and harvested in RIPA buffer(50mM Tris-HCl pH8.1, 150mM Nacl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor). Total protein were measured using the bicinchonic acid(BCA) assay(Pierce, Rockford, IL, USA). Protein samples were loaded and separated on SDS-polyacrylamide gels, transferred to PVDF membrane(Whaman Iternational, Ltd., Maidstone, UK), probed with specific primary antibodies, washed and probed with secondary antibodies. Signals were detected using an enhenced chemiluminescent substarate(WEST-ZOL, iNtRON Biotechnology Inc, Seoul, Korea). The primary antibodies used include Hep B xAg, Cyclin

Collection @ jeju

D1, Cyclin E, Survivin , Cdk2, Cdk4, cyclin A, cyclin B1(Santa Cruz Biotech, CA, USA), and GAPDH, p21, Cleaved PARP, Bax, Bcl-2(Cell Signaling Technology, MA, USA), p27(BD Biosciences, CA, USA).

3.4.Cell cycle analysis by flow cytometry

The HBx stable huh7 and Chang cells were synchronized using serum-free medium for 24hr and stimulated by replacing complete medium. DNA content was assessed by staining ethanol-fixed cells with propidium iodide (SIGMA, St.Louis, MO, USA) and measured by FACS (BD biosciences, San Jose, CA, USA). The percentage of each phases of cell-cycle analysis was perform using Cell Quest Pro software (BD Biosciences). Proliferation index (PI)=(G2M+S)/(G0G1+S+G2M)*100 was calculated.

3.5. Intracellular localization

Cells were counterd and $8x10^4$ per well were seeded onto 35x10mm cell culture dish before the day of transfection. pEGFP-N1 and Recombinant pEGFP-HBx plasmids were transfected into huh7(human hepatoma cell line), Chang(human normal liver cell line)cells. At 24hr post-transfection, we observed subcellular localization of HBx through a fluorescence microscopy (IX70: Olympus, Tokyo, Japan).

Collection @ jeju

3.6. RT-PCR

Total RNA was isolated from each cells using Trizol reagent(Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized from 1ug of total RNA using Reverse transcription system (Promega, Madison, WI, USA). PCR primers for amplification of HBx : Forward 5'-CTCTACCGTCCCCTTCTTCG-3' , Reverse 5'-AATCTCCTCCCCCAACTCCT-3' , β-actin : Forward 5'-GTGGGCCGCCTAGGCACCA-3', Revers 5'-GGAGGAAGAGGATGCGGCAG-3' , TGF-β1 : Forward 5'-GTATTTCTGGTACAGCTCCA , Reverse 5'-GTTCAAGCAGAGTACACACA-3' . PCR was performed using Taq polymerase(iNtRON Biotechnology Inc, Seoul, Korea). HBx, β-actin, TGF- β PCR conditions were with 5min at 95℃, 30 sec denaturation at 55℃ and HBx was 40 cycle of annealing at 55 ℃ for 40sec, β-actin was 25 cycle of annealing at 56℃, TGF-β was 35 cycle of annealing at 52℃ and than 1min elongation at 72℃. RT-PCR products were analyzed by electrophoresis in 1% agarose gels contaninng 0,002% Nucleic acid staining solution(RedSafe TM, Biotechnology Inc, Seoul, Korea).

Collection @ jeju

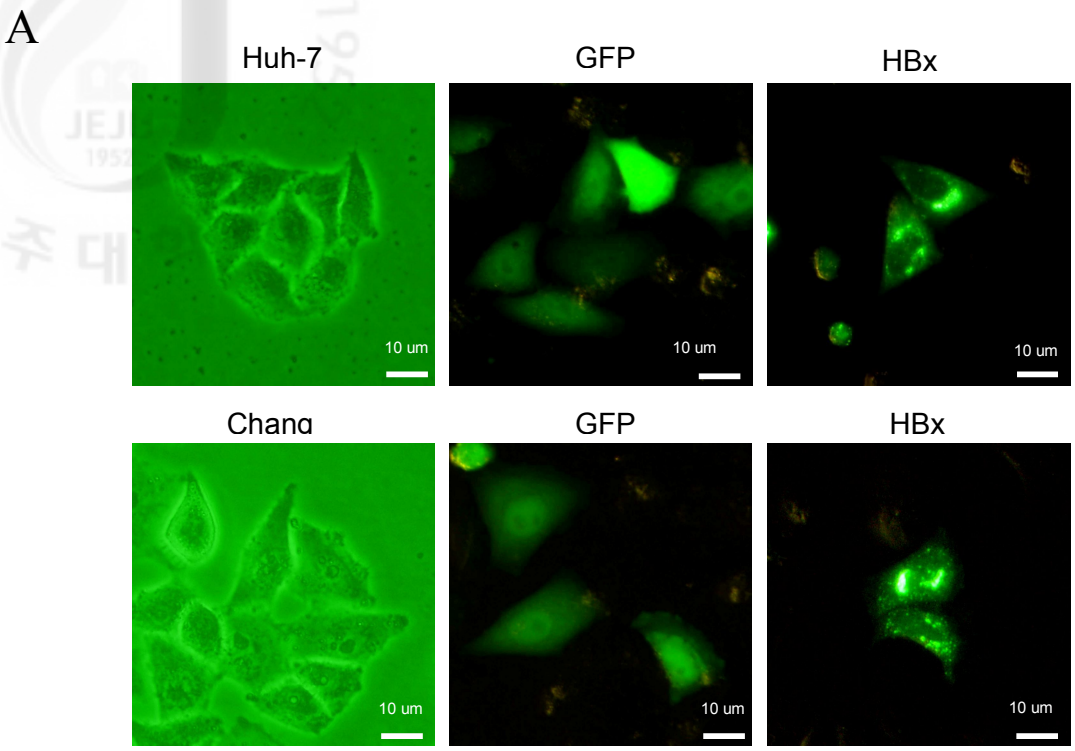
4. RESULTS

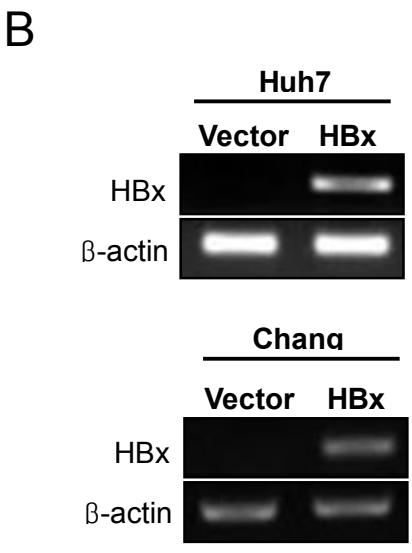
4.1. HBx protein was localized in cytoplasm and around the nuclear membrane in Huh7 and Chang cell lines.

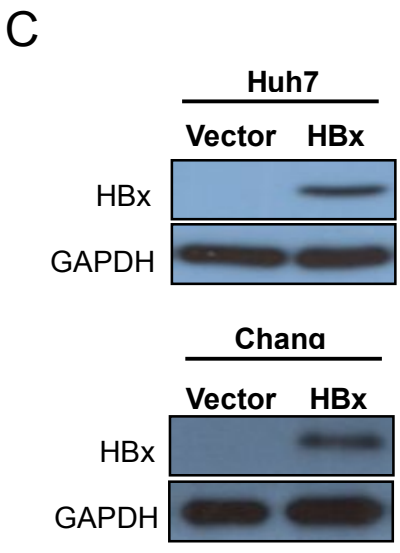
GFP-fused HBx plasmids were transient transfected for 24hr in human normal liver Chang cells and human hepatocellular carcinoma Huh7 cells and observed the expression of GFP-HBx and its localization using the fluorescent microscope. HBx was mostly localized in cytoplasm and expressed in round the specific regions of nuclear membrane. Intracellular localization of HBx was not different between Huh7 and Chang cells (Fig. 1 A and B).

To investigate the role of HBx , Cells were stably transfected with the HBx gene(Huh7-X ,Chang-X)and HBx expression was analyzed by RT-PCR and western blot (Fig. 1 C and D). The expression of cell cycle regulatory proteins in HBx stably expressed Huh7-X and Chang-X cell lines were investigated by western blot analysis.

Collection @ jeju







dCollection @ jeju

Figure 1. Intracellular localization of HBx in Huh7 and Chang cells and HBx mRNA and protein expression in HBx stable huh7(Huh7-X) and chang(Chnang-X)cells. (A and B) pEGFP-N1 vector and GFP-fused HBx plasmids were transiently expressed in Huh7 and Chang cells for 24hr and examined the intracellular localization of HBx using the fluorescent microscope (x200). (C and D) Huh7 and Chang cell were transfected with HBx gene and G418-resistant clones were selected and HBx expression was analyzed by RT-PCR and western blot. B-actin and GAPDH were used as a control for equal loading of mRNAs and proteins.

Collection @ jeju

4.2. HBx differently regulate the expression of cyclin-dependent kinase inhibitor p27 proteins and cell cycle progression in Huh7-X and Chang-X cells.

Hepatitis B X protein is known as intracellular mutifuntional regulatory protein and it regulates the cell cycle progression or suppression through regulation of cell cycle proteins and various intracellular signaling pathways. However, role of HBx is controversial in the cell cycle regulation [4, 8, 9, 14-17, 24, 25].

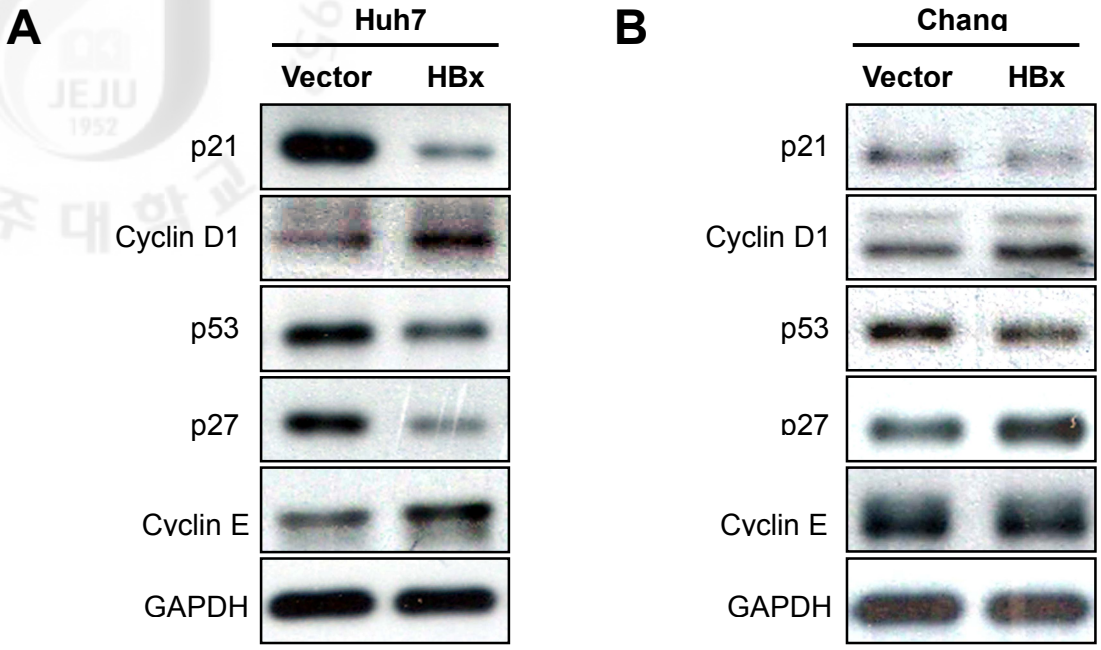
As shown in Figure 2A, negative cell cycle regulators such as cyclin-dependent kinase inhibitor p21 and p27 were dramatically down-regulated in Huh7-X cell line compared to Huh-V cells.. Also, expression level of p53 protein which is known as p21 inducer was down-regulated. In addition, expressions of cell cycle activator proteins such as Cyclin D1, Cyclin E, were up-regulated (Fig. 2A). But in the Chang-X cell line, expression of p21 and p53 were slightly down-regulated and expressions of Cyclin D1 and E were not changed significantly. However, expression level of p27 was highly up-regulated (Fig. 2 B and D). These results indicate that HBx differently regulate the cyclin-dependent kinase inhibitor p21 and p27 in Huh7 and Chang cells (Figure 2. C and D) and it may affect the cell cycle progression in Huh7 and Chang cells.

To determine the effects of HBx on cell cycle progression in Huh7 and Chang cells, we measured cell cycle by propidium iodide(PI) staining and flow cytometry (FACS). The

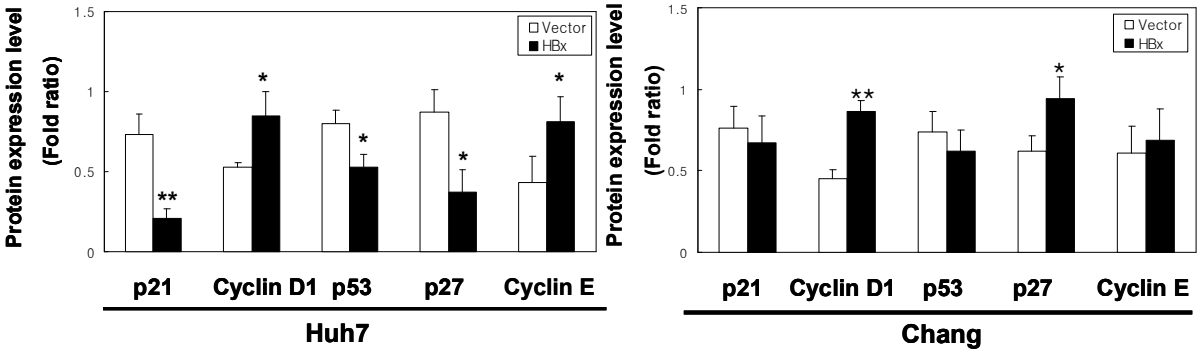
dCollection @ jeju

proportion of G0/G1 phase in Huh7-X cells were decreased more than the Huh7-V cells (60.62% for Huh7-V vs 53.77% for Huh7-X, Fig. 2 D). The proliferation index (PI) were calculated using the formula (PI=(G2M+S)/(G0/G1+S+G2M)*100). A PI of Huh7-X cells was more increased than the Huh7-V cells (46.49% for Huh7-X, 39.54% for Huh7-V, Fig. 2 D). These results demonstrated that proliferation was enhanced in Huh7-X cells. On the other hand, in the Chang cells, G1/S progress was arrested more in Chang-X cells than Chang-V cells (61.36% for Chang-V vs 63.65% for Chang-X, Fig. 2 E). Moreover, PI was more decreased in Chang-X than the Chang-V cells (Fig. 3 E), which demonstrated that proliferation was attenuated in Chang-X cells. This result suggests that HBx may play a differential role in hepatoma and normal liver cells.

Collection @ jeju



C



D

% Cell cycle Distribution	Huh7		Chang	
	Vector	HBx	Vector	HBx
G0/G1(%)	60.62±0.15	53.77±0.31	61.36±0.52	63.65±0.2
S(%)	17.23±0.18	19.7±0.25	14.84±0.38	15.01±0.48
G2/M(%)	22.41±0.2	27.01±0.08	24.25±0.8	21.72±0.38
PI(%)	39.54±0.24	46.49±0.32	38.92±0.54	36.59±0.23

dCollection @ jeju

Figure 2. Expression of cell cycle regulatory proteins and different cell cycle regulation in Huh7-X and Chang-X cells. Proteins extracted by RIPA cell lysis buffer and proteins were analyzed by western blot. (A) in Huh7-X, (B) in Chang-X cells.(C) The histogram shows the results from applying Image J program. Values shown represent the mean ± s.d. of three indepenent experiment. *P<0.05 and **P<0.01 versus control(Huh7-V or Chang-V). (D and E) Cell cycle was examined by PI staining and flowcytometry (n=3) in each cells and analyzed by Cell Quest Pro Software. Proliferation index (PI)=(G2M+S)/(G0G1+S+G2M)*100 was calculated. Values shown represent the mean ± s.d. of three indepenent experiment.

Collection @ jeju

4.3. TGF-β mRNA expression was down-regulated in Huh7-X cells and up-regulated in Chang-X cells.

To future investigate how HBx regulate p27 expression; we examined the intracellular expression level of TGF-β mRNA by RT-PCR analysis. TGF-β is a multifunctional cytokine and regulates the cyclin-dependent kinase inhibitor such as p21, p27, p15. Since TGF-β induce the G1 phase arrest through p27(kip1) up-regulation [26, 27], we presumed that HBx may differently regulate p27 expression via expression of the TGF-β. The RNA in each cells were isolated and the expression levels of TGF-β mRNA were investigated, expression of TGF-β mRNA was down-regulated in Huh7-X cells (Fig. 3 A and C), In the Chang-X cells, expression of TGF-β mRNA was up-regulated (Fig. 3 B and C). This result suggests that in the Huh7 cells, HBx down-regulate the p27 expression and promote cell cycle progression through down-regulation of TGF-β. However, in the Chang cells, HBx inhibit p27 expression and cell cycle progression through up-regulation of TGF-β.

It has been reported that HBx shifts TGF-β signaling from tumor-suppressive TBRI/pSmad3C/p21 pathway to activated-JNK/pSmad3L/c-Myc oncogenic pathway in early carcinogenic process of HBV-infected hepatocyte [23]. Our previous result showed that HBx differently regulates the expression of TGF-β mRNA level in Huh7-X and Chang-X cells. Base on this result, we presumed that HBx may differently affect the activated-

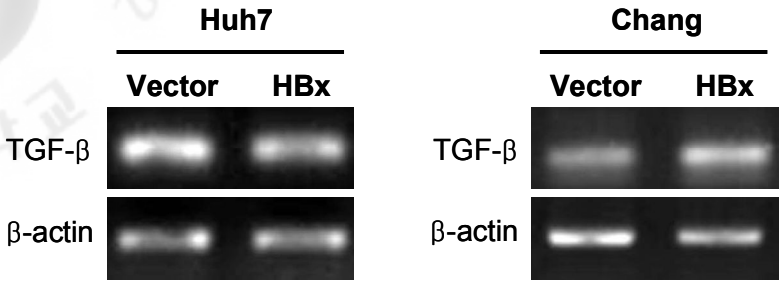
Collection @ jeju

JNK/pSmad3L/c-Myc oncogenic pathway via regulation of TGF-β expression in Huh7 and Chang cells and we analyzed expression of proteins of activated-JNK dependent oncogenic pathway by western blot.

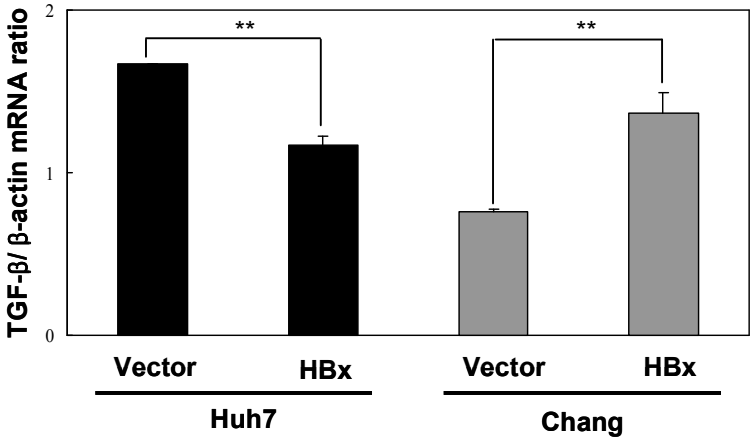
Basal expression level of oncogenic pathway proteins such as pJNK and c-myc more increased in Huh7 cells than Chang cells. However, contrary to our expectations, HBx didn't directly affected regulation of pSmad3L oncogenic signaling pathway proteins (Fig. 3 C).

Collection @ jeju

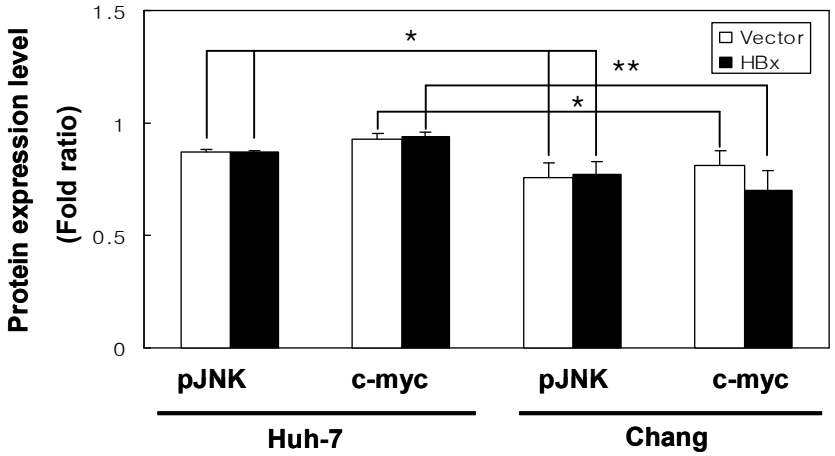
A



B



C



dCollection @ jeju

Figure 3. Expression of TGF-β mRNA in Huh7-X and Chang-X cells. Expression levels were measured by RT-PCR. The amplified TGF-β DNA was separated by electrophoresis through 1% agarose gel and visualized by staining (A). Histogram showing results of data analysis by the Image J program. (B). Values shown represent the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. control (Huh7-V or Chang-V). Proteins involved in oncogenic pathway were compared by western blot analysis in Huh7 and Chang cells with/without HBx expression (C). Values shown represent the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. Chang cells.

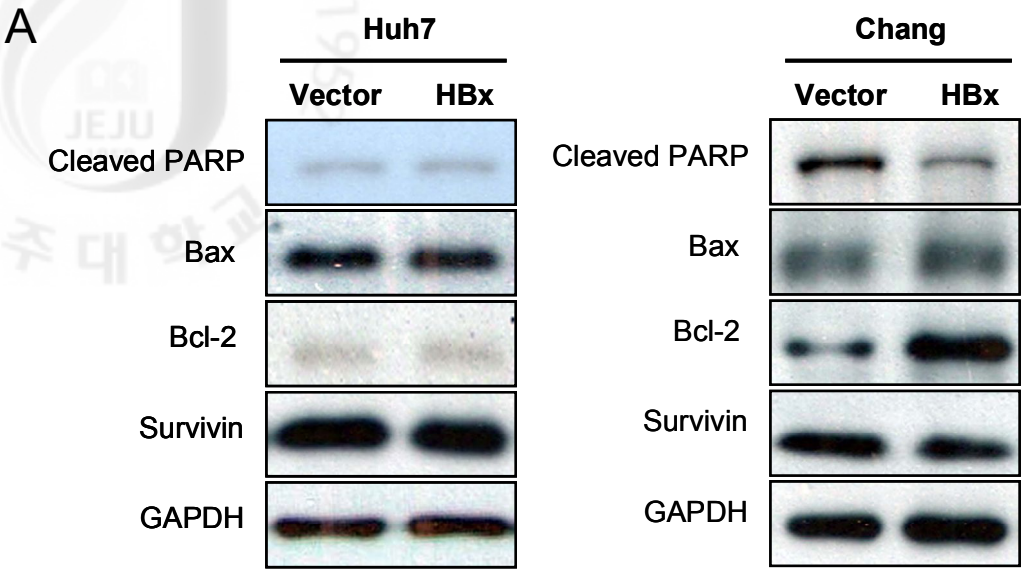
Collection @ jeju

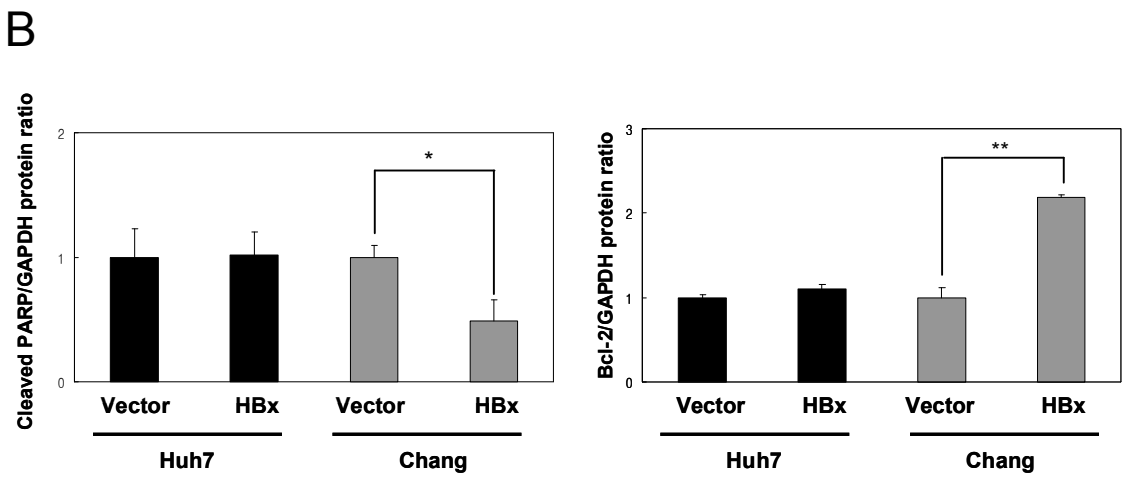
4.4. HBx inhibit production of cleaved PARP as a marker for apoptosis protein via up-regulation of Bcl-2 in Chang cells.

To investigate the relationship between HBx and cell death, we examined the expression of apoptosis regulatory proteins by western blot. When comparing Huh7-V and Huh7-X cells, apoptotic proteins such as Bax and Cleaved PARP or anti-apoptotic proteins such as Bcl-2 and Survivin expression did not change (Fig. 4 A and C). However, Chang-X cells the expression of Bcl-2 protein is up-regulated than Chang-V control cells and Cleaved PARP protein level decreased in Chang-X cells dramatically (Fig. 4 B and C). These results indicated that HBx expression influence the apoptosis as an anti-apoptotic factor only in normal liver cells.

However, in unfavorable condition, HBx also work as an anti-apoptotic factor in hepatoma cell line. The cells were cultured in serum-free media for 72hr and induced the serum starvation mediated apoptosis. In starvation condition, production of cleaved PARP was inhibited in Chang-X cells and more in Huh7-X cells (Figure 4. D and E). In Huh7-X cells, expression of p53 was inhibited and Bcl-2 was slightly up-regulated.

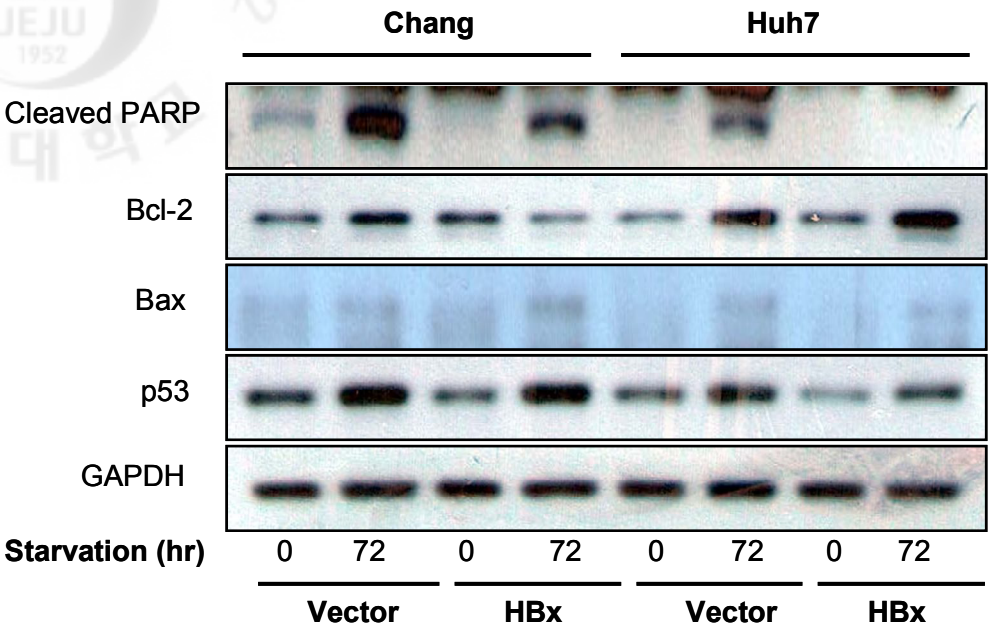
Collection @ jeju



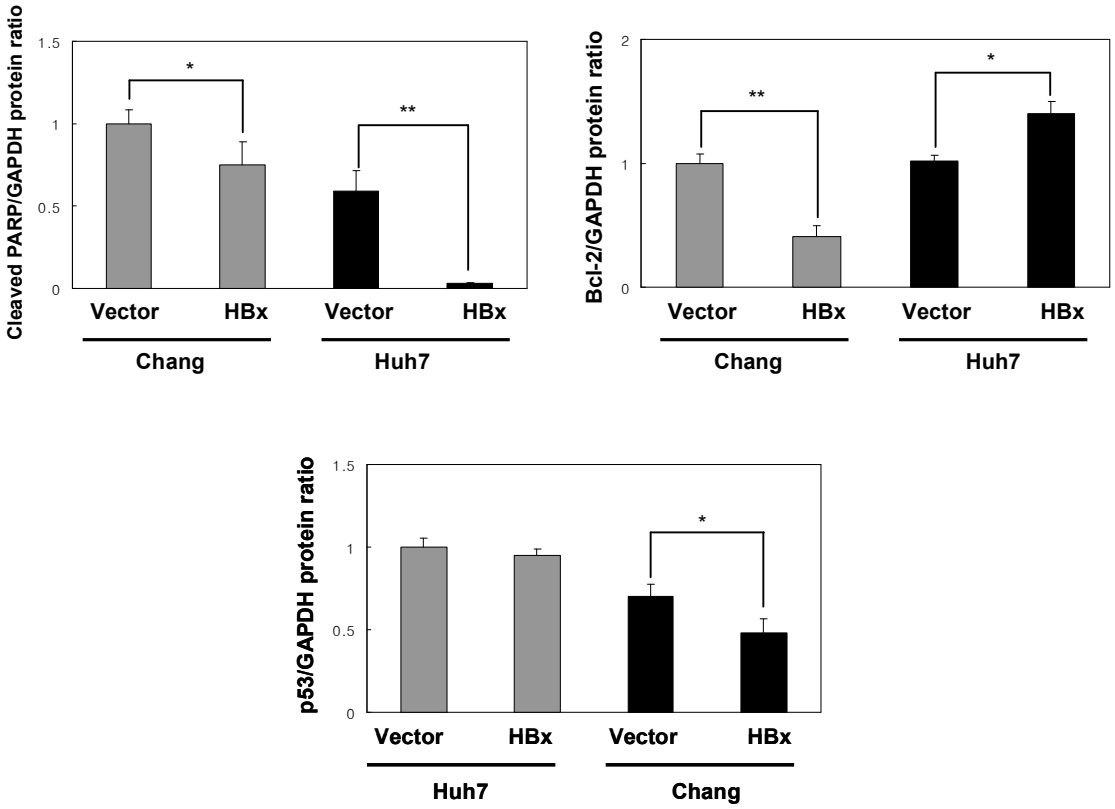


dCollection @ jeju

C



D



dCollection @ jeju

Figure 4. Expression of apoptosis-related proteins in Huh7-X and Chang-X cell lines. Protein levels were analyzed by western blot using specific antibodies. (A) in Huh-7 (B), in Chang-X cells. (D) Each cell have cultured with 10% FBS medium for 24hr and changed to serum-free medium for 72hr. After cell lysis, proteins were analyzed by western blot. (C and E) The histogram shows the results from applying Image J program. Values shown represent the mean ± s.d. of three indepenent experiment. *P<0.05 and **P<0.01 versus control(Huh7-V or Chang-V).

Collection @ jeju

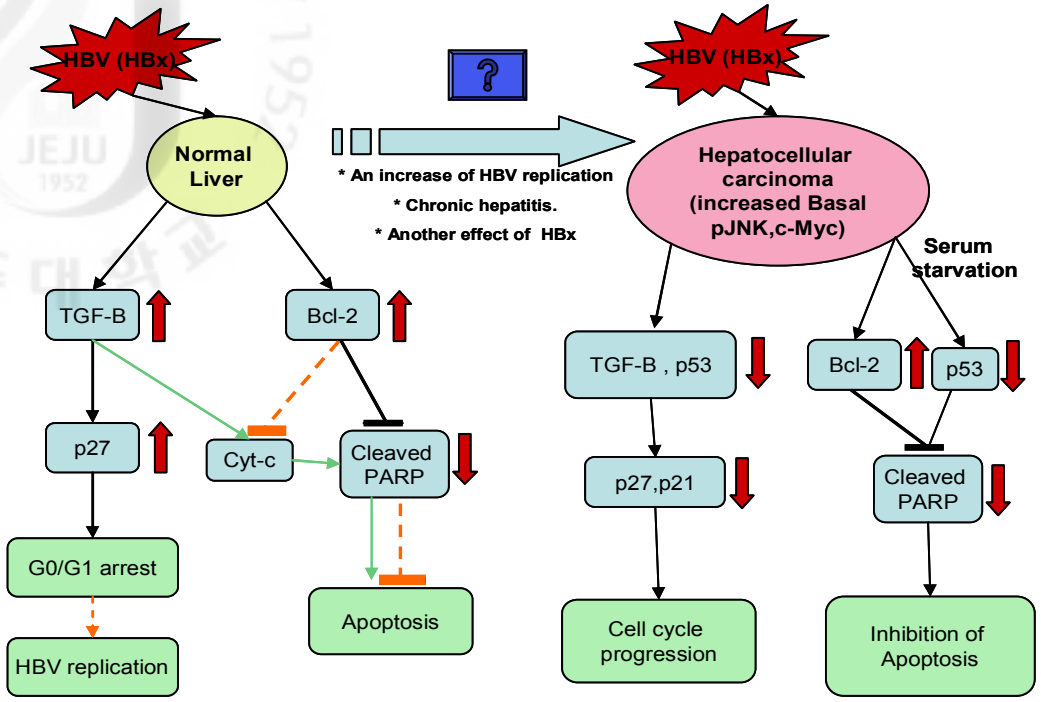


Figure 5. Proposed model for the different effects of HBx in hepatoma cell and normal liver cell lines. (A) HBx induce the G1 phase arrest of cell cycle via increase TGF-B and p27 expression and which prevent the TGF-B mediated apoptosis pathway by the increase of Bcl-2 expression in HBV infected normal liver cells. Previous report showed that HBV replication was increased by the G1 or G2 phase arrest. We suggest that HBx-mediated an increase of TGF-B, p27 expression and cell cycle arrest was required for the HBV replication. HBx also prevent TGF-B mediated apoptosis via an increase of Bcl-2 expression for the cell survival during HBV replication in normal liver cells. (B) we also suggest that HBx probably will more stimulate development of HCC via cell cycle acceleration on the part of a down-regulation of TGF-B and p53 and inhibit the apoptosis in unfavorable condition such as serum starvation in hepatocellular carcinoma (HCC) cells.

Collection @ jeju

5. DISCUSSION

In the present study, we evaluated the intracellular roles of HBx on the impact of cell cycle regulation and apoptosis in Chang and Huh7 cell lines. Our results have demonstrated that HBx differently regulated the cell cycle progression and apoptosis in each Chang and Huh7 cell (Fig. 2 and 4). Considering that Chang cell is a normal liver cell line with HeLa markers[13]and Huh7 is a well differentiated hepatocyte derived cellular carcinoma cell line , our findings may provide some clues at the controversial issue of intracellular roles of HBx.

The change of cell cycle regulatory proteins and modulation of cell cycle by HBx have been studied on various cell lines. HBx up-regulated the expression of DNA methyltransferase 1 through a regulatory circuit involving the p16-cyclin D1-CDK 4/6-pRb-E2F1 pathway in HepG2 hepatoma cells[16]and it induced cell cycle progression via up-regulation of β-1,4-galactosytransferase I[28]. HBx also promoted G1/S transition of cell cycle through interact with cyclin E/A-cdk2 complex and p27 destabilization in Huh7 and HEK-293 human hepatoma cells [14]. H7402 stably expressed HBx (H7402-X) down-regulated the expression of p27, but up-regulated expression of the Cyclin D, Cyclin E and proliferation index(PI)[4]. In contrast, expression of HBx increased the cell cycle regulatory proteins such as p27 and p21 via a calcium-dependent manner in primary human

Collection @ jeju

hepatocytes[24]. In Chang cells expressing HBx(Chang-X), HBx repressed cell growth and reduced the expression of Cyclin D1, Cyclin E, Cyclin B1, CDK2 and CDK4[11]. Moreover, HBx arrested the G1 phase of cell cycle and inhibited the tumorigenicity in Chang-X cell line[13]. Interestingly, in our results indicated that HBx especially down-regulate the expression of p27 and promoted G1/S transition of cell cycle in human hepatoma Huh7 cells, while it down regulate the expression of p27 and arrested the G1 phase of cell cycle in human normal liver Chnag cells(Fig. 2).

Transforming growth factor β1 (TGF- β1) has been reported that it induced the G1 phase arrest via p27(kip1) up-regulation[27]. It has reported that expression of HBx attenuated growth inhibition response to TGF-β 1 in Mink lung epithelial cells (Mv1Lu cells)[29]. In this study, HBx decrease the mRNA expression level of TGF-β in Huh7 cells. However, it increases that in Chang cells (Fig. 3). These findings have showed the possibility that HBx may differently regulate cell cycle via modulation of p27 by TGF-β in Huh7 and Chang cells.

The expression of HBx differentially affected the production of TGF-β between normal and hepatocarcinoma cells. In the hepatocarcinoma cells such as Huh7 cells, HBx decrease the level of TGF-β mRNA and it may alleviate the G1 arrest caused by TGF-β. But in early infection, constitutive expression of HBx in normal liver cell increased TGF-β expression

Collection @ jeju

and arrest cell cycle to give favor for HBV replication. The excess TGF-β by HBx in normal liver cells may lead shift TGF-β signal from tumor suppression to oncogenesis.

Recently, it was reported that HBx shifts TGF-β signaling from tumor suppression to oncogenesis in early carcinogenic process [23]. In the JNK/p38 non-Smad pathway, TGF-β receptors interact with TRAF6 and activate JNK/p38[30]. They suggested that HBx participated directly in hepatocarcinogenesis by shifting hepatocytic smad3-mediated signaling from tumor suppression to oncogenesis in patients with early chronic hepatitis B. In Huh7 cells, the level of TGF-β is higher than normal cell and the JNK-dependent pathway is already activated and the HBx expression did not increase more activation of JNK-dependent signaling (Supplementary Fig. 1). Once the normal cells turn into hepatoma cells, function of HBx may change to assisted to proliferation and anti-apototsis.

HBx mediated cell death is another controversial issue and it was known that induction of apoptosis by HBx occurred via three primary pathways such as death receptor, mitochondria and p53 dependent apoptosis [3]. The HBx was also reported to inhibit the apoptosis via inhibition of p21, p27 expression and up-regulation of anti-apoptotic proteins such as Bcl-2 and Survivin[4, 9, 17, 18]. Interestingly, HBx inhibited apoptosis via up-regulation of Bcl-2 despite an inhibition of cell cycle progression in Chang cells (Figure 4) and our result has indicated that expression of TGF-β was increased by HBx in Chang cells (Figure 3). TGF-β

Collection @ jeju

has been reported that it induce the mitochondria dependent apoptosis through NOX4 mediated ROS production [31, 32]. Bcl-2 is recognized as an anti-apoptotic protein that acts as an inhibition of mitochondrial cytochrome C release. Taken together, our results suggest that up-regulation of Bcl-2 by HBx may interrupt production of cleaved PARP through inhibition of cytochrome C release and activation of caspase. That inhibits the TGF-β mediated cell death in Chang cells where TGF-β is increased. Meanwhile, HBx did not affect the apoptosis in Huh7 cells, however, we has found that HBx inhibited the apoptosis in unfavorable condition such as serum starvation (Figure 4). When the expression of HBx was inhibited by specific sh RNA in HBV infected hepatoma cells such as HepG2.2.15 cells, apoptosis was increased[33].

In this study, we suggest dual function of HBx in different stage of infection, normal hepatocyte, HBx may inhibit cell cycle progression via TGF-B-mediated p27 up-regulation for HBV genome replication and apoptosis via up-regulation of Bcl-2 to support the HBV replication. In chronic hepatitis, HBx shift TGF-β signal from tumor suppression to oncogenesis[23]. When the character of cell has changed from the hepatocyte to hepatoma, HBx may more play oncogenic character via promotion of cell cycle progression and suppression of apoptosis.

Collection @ jeju

JEJU NATIONAL UNIVERSITY 1952
JEJU 1952
제주대학교

PART Ⅱ

The mutation of the hepatitis B virus X gene modulate the cell cycle and apoptotic proteins in Huh-7 cells

.

dCollection @ jeju

1. ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers. HCC and HCC–associated disease are caused by hepatitis B virus (HBV). HBV often occur mutation at basal core promoter (BCP), which is overlapped with open reading frame (ORF) of hepatitis B virus X (HBx) protein. The HBx protein plays very important role in development of liver cancer. In this study, we have investigated the influence that a mutation of HBx (BCP1) had on to cell cycle in Hepatocellular carcinoma cell line, Huh-7. We has cloned mutant of HBx from clinical samples, and then transiently and stably transfected into Huh-7. The mutant HBx (BCP1) protein especially reduced expression of CDK inhibitor p27kip1 and more promoted the cell cycle progression than HBx. BCP1 also inhibited the production of cleaved PARP as a marker for apoptosis than HBx in Huh7 cells. In addition, multifunctional cytokine TGF-β was more down-regulated by BCP1 mutant. Therefore, BCP1 mutation more promotes the cell cycle progression and inhibits the apoptosis than HBx in Huh-7 cells, which may require the down-regulation of TGF-β.

***Key words*:** HBx , HBV, cell cycle, apoptosis, TGF-β

dCollection @ jeju

2. INTRODUCTION

Infection of hepatitis B virus(HBV) is a major cause of HCC-associated disease including liver cirrhosis(LC), acute hepatitis B(AHB), Chronic hepatitis B(CHB) [1]. Approximately 2 billion people infected with HBV over the world and more than 400million people are chronic HBV carriers [2]. It is predominantly found in the Eastern Asia and south Africa and hepatocellular carcinoma(HCC) is a malignant tumor of third high occurrence frequency in South Korea [3].

The HBV have a 3.2 Kb length relaxed circular double stranded DNA genome and it contains a highly compact genetic organization with four overlapping open reading frames(ORF) including surface gene(preS1/S2/S), core gene(pre-C/C), polymerase gene(P) and X gene [4, 5].

Clinical studies have indicate that the mutation of HBV basal core promoter (BCP) region increase the risk of hepatocelluar carcinoma(HCC) [1, 6-8]. BCP regulate the transcription of precore and pregenomic mRNA [9] and mutations of BCP enhance HBV replication [10, 11] and the most common BCP double mutation (A1762T and G1764A nucleotide exchange) decrease the HBe Ag expression above 70% but enhanced viral genome replication [12]. Moreover, An additional mutation of BCP region (T1753C or C1766T nucleotide exchange)

Collection @ jeju

goes with BCP double mutation (A1762T and G1764A nucleotide exchange).These mutants more promote the HBV replication than the BCP double mutation and increase the replication above eight times compared with wide type HBV, but expression of HBe Ag has been decreased [9]. However, clinical studies have indicated that basal core promoter mutation is not always associated with high serum HBV DNA levels [13-15].

The hepatitis B virus x (HBx), a small protein of 154 amino acids, is mostly localized in cytoplasm, nucleus and mitochondria [16] and it is essential for early virus infection, HBV genome replication and plays a crucial role in the HBV associated diseases and development of hepatocellular carcinoma(HCC) [17, 18]. HBx does not bind to DNA, but it is able to activate the transcription factors, such as NF-kB, CREB, AP-1, AP-2, SP-1 and it activate the proto-oncogenes, such as c-Fos, c-myc, c-Jun, EGF [3].

HBx is also modulates a variety of host signaling pathways including Ras-Raf-MAPK/ERK, JNK, JAK/STAT, PI3-K/AKT, Wnt/B-catenin and it regulate a proliferation, apoptosis , differentiation, matastasis of cancer cells [2, 3]. Previous studies demonstrated that HBx promote the cell cycle progression via modulation of negative cell cycle regulators such as p16, p21, p27, p53 and progressive regulators such as Cyclins, CDKs , pRb. HBx also inhibit an apoptosis via up-regulation of anti-apoptotic proteins bcl2 and survivin proteins [18-28].

Collection @ jeju

The C-terminal region of the hepatitis B virus X protein is overlapped with HBV basal core promoter(BCP) region and HBx mutation often occur together with BCP mutation [3, 11]. Most of previous studies examined BCP mutation regarding HBV replication, but the molecular mechanism of mutation of HBx with corresponding to BCP mutation has not been enough examined.

In this study, we hypothesized that HBx may be promote the cell cycle progression and inhibit apoptosis through mutation at C-terminal of HBx with corresponding to BCP mutation and we has been examined a role of HBx mutant (BCP1) on the modulation of cell cycle and apoptosis in human hepatocellular carcinoma Huh7 cell lines.

Collection @ jeju

3. MATERIALS AND METHODS

3.1. Cell culture and transfection

Huh-7(human hepatoma cell line) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum(FBS)(GIBCO inc, Grand Island, NY, USA). Huh-7 cells were transfected with pEGFP-HBx plasmid . For transfection, Lipofectamine 2000(Invitrogen) is used accoding to manufacturer's protocols. For stable transfection, 24hr after transfection, transfected cells were selected by G418(500ug/ml, AG Scientific, CA, USA) containing medium. The individual G418-resistant clones were selected and analyzed by PCR and westhern blot

3.2. Plasmids

The HBx and HBx mutant(BCP1) genes cloned into pGEM was donated by Dr. Song Byoung-Cheol (Cheju University , Cheju, Korea). A recombinant plasmid was made by HBx into the pEGFP-N1 plasmid vector. The insert was amplified by PCR using a pGEM template containing HBx with addition of XhoI and EcoRI restriction sites to primers matching those found in the pEGFP-N1. The following primers(Integrated DNA Technologies) were used: HBx GFP-XhoI Forward 5'-

Collection @ jeju

ATCCTCGAGATGGCTGCTCGGGTGTGC-3' , HBx GFP-EcoRI Reverse 5'-GAT GAATTCCGGCAGAGGTGAAAAAGTTGC-3' Restriction digest with XhoI and EcoRI-HF enzymes(New England Biolabs,Ipswich,MA,USA) was performed for both the insert and vector, followed by ligation with T4 DNA ligase(Promega, Madison, WI, USA).

3.3. Western blots and antibodies

Cells transfected with indicated plasmids and harvested in RIPA buffer(50mM Tris-HCl pH8.1, 150mM Nacl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor). Total protein were measured using the bicinchonic acid(BCA) assay(Pierce, Rockford, IL, USA). Protein samples were loaded and separated on SDS-polyacrylamide gels, transferred to PVDF membrane(Whaman Iternational, Ltd., Maidstone, UK), probed with specific primary antibodies, washed and probed with secondary antibodies. Signals were detected using an enhenced chemiluminescent substarate(WEST-ZOL, iNtRON Biotechnology Inc, Seoul, Korea). The primary antibodies used include Hep B x Ag, Survivin (Santa Cruz Biotech, CA, USA), and GAPDH, p21, Cleaved PARP, Bax, Bcl-2(Cell Signaling Technology, MA, USA), p27(BD Biosciences, CA, USA).

Collection @ jeju

3.4. Cell cycle analysis by flow cytometry

Huh7 cells were transiently transfected with HBx or BCP1 mutant and, 4h post-transfection, changed to serum-free medium for 24hr. HBx or BCP1 mutant stable huh7 cells (Huh7-X and Huh7-BCP1) were synchronized using serum-free medium for 24hr and stimulated by replacing complete medium. DNA content was assessed by staining ethanol-fixed cells with propidium iodide (SIGMA, St.Louis, MO, USA) and measured by FACS (BD biosciences, San Jose, CA, USA). The percentage of each phases of cell-cycle analysis was performed using Cell Quest Pro software (BD Biosciences). Proliferation index (PI)=(G2M+S)/(G0G1+S+G2M)*100 was calculated.

3.5. Intracellular localization

Cells were counterd and $4x10^5$ per well were seeded onto 60x15mm cell culture dish before the day of transfection. pEGFP-N1 and Recombinant pEGFP-HBx and HBx-BCP1 plasmids were transfected into huh7(human hepatoma cell line). At 24hr post-transfection, MitoSOX (Molecular Probes, Invitrogen, USA) was pre-treated to HBx or BCP1-transfeced cells according to manufacturer's instructions and incubate at 30 °C for 10 min, washed once using the filtered PBS. We observed subcellular localization of HBx and mitochondria through a fluorescence microscopy (IX70: Olympus, Tokyo, Japan).

Collection @ jeju

3.6. RT-PCR

Total RNA was isolated from each cells using Trizol reagent(Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized from 1ug of total RNA using Reverse transcription system(Promega, Madison, WI, USA). PCR primers for amplification of HBx : Forward 5'-CTCTACCGTCCCCTTCTTCG-3' , Reverse 5'-AATCTCCTCCCCCAACTCCT-3' , β-actin : Forward 5'-GTGGGCCGCCTAGGCACCA-3', Revers 5'-GGAGGAAGAGGATGCGGCAG-3' , TGF-β1 : Forward 5'-GTATTTCTGGTACAGCTCCA , Reverse 5'-GTTCAAGCAGAGTACACACA-3' . PCR was performed using Taq polymerase(iNtRON Biotechnology Inc, Seoul, Korea). HBx, β-actin, TGF- β PCR conditions were with 5min at 95℃, 30 sec denaturation at 55℃ and HBx was 40 cycle of annealing at 55 ℃ for 40sec, β-actin was 25 cycle of annealing at 56℃, TGF-β was 35 cycle of annealing at 52℃ and than 1min elongation at 72℃. RT-PCR products were analyzed by electrophoresis in 1% agarose gels contaninng 0,002% Nucleic acid staining solution(RedSafe TM, Biotechnology Inc, Seoul, Korea)

Collection @ jeju

4. RESULTS

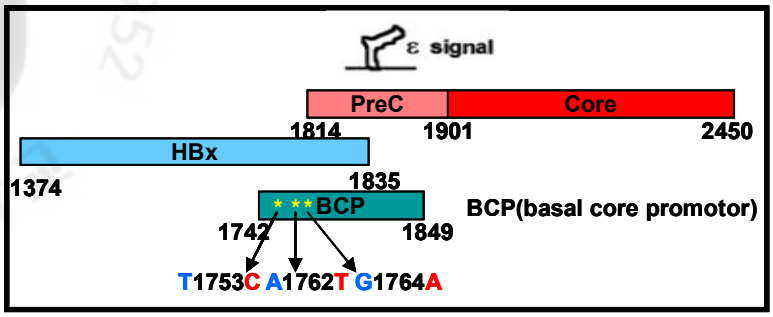
4.1. Intracellular localization of HBx and BCP1 were not difference and localized in cytoplasm and around the nuclear membrane in Huh7 and Chang cell lines.

Figure 1A showed the location of mutation found in clinical samples (BCP1, A1762T G1764A, T1753C nucleotide exchange; I127T, K130M, V131I amino acids changes).

The intracellular localization of HBx mostly localized in cytoplasmic, with a partial fraction in the nucleus [16] and mitochodria [18, 29]. GFP-fused HBx or BCP1 mutant plasmids were transient transfected for 24hr in human hepatocellular carcinoma Huh7 cells and human normal liver Chang cells and observed the expression of each protein. Its localization was observed using the fluorescent microscope. HBx or BCP1 mutant were primarily localized in cytoplasm and expressed with punctuated pattern in a round the specific regions of nuclear membrane. Co-staining with Mitosox, the mitochondria specific dye revealed that the punctuated region indicated mitochondria. However, Intracellular localization of HBx or BCP1 mutant was not difference in Huh7 or Chang cells (Fig.1B).

Collection @ jeju

A



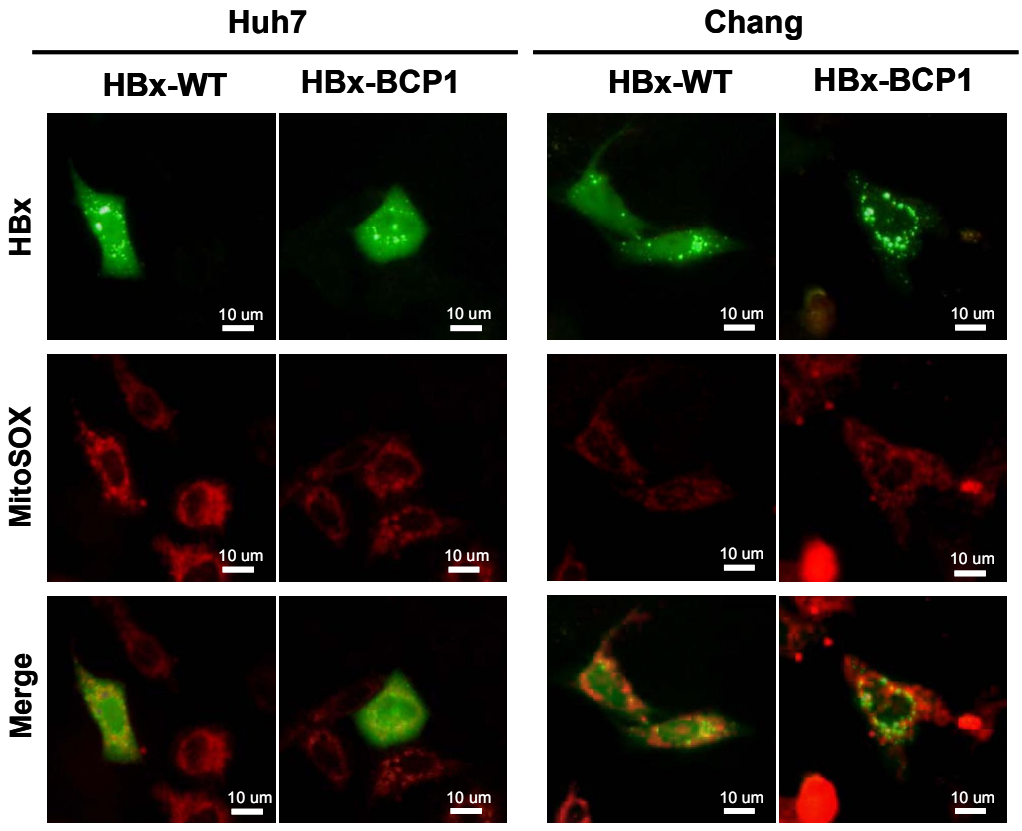
<Nucleotide>

	1	380	389 391	465
HBx W T	ATGGCTGCTCGGGTG---------	ATTAGGTTAAAGGTC---GCCTAA		
HBx BCP1	ATGGCTGCTCGGGTG---------	ACTAGGTTAATGATC---GCCTAA		

<Protein>

	1	127 130131	154
HBx WT	MAARVCCQLDPAR--------E	IRLKVFVLGGCRHKLV------NFFTSA	
HBx BCP1	MAARVCCQLDPAR--------E	TRLMIFVLGGCRHKLV------NFFTSA	

B



dCollection @ jeju

Figure 6. Schematic illustration of the HBx or BCP1 mutant and its intracellular localization in Huh7 and Chang cells. The position of mutation in HBx gene and HBV genome is indicated. The altered amino acids are indicated below (A). pEGFP-N1 vector and GFP-fused HBx or HBx-BCP1 mutant plasmids were transiently expressed in Huh7 and Chang cells for 24hr with Mitosox (red) and examined the intracellular localization of HBx (green) using the fluorescent microscope (x 200)(B).

Collection @ jeju

4.2. BCP1 mutant especially down-regulate the expression of cyclin-dependent kinase inhibitor p27 proteins and promotes the cell cycle progression than HBx in Huh7 cells.

Hepatitis B X protein (HBx) is known that it promotes the cell cycle via modulation cell cycle regulatory proteins such as p16, p21, p27, p53, Cyclins and CDKs [19-21]. HBx core promoter mutation was down-regulated the p21 by deregulation SKP2[30] (32).

To investigate the role of HBx and HBx mutant (BCP1) in cell cycle regulation, Huh7 cells were stably transfected with HBx and BCP1 mutant genes. Expression of HBx or BCP1 mutant and cell cycle regulatory proteins were investigated by western blot analysis (Figure 2A). As shown in Figure 2A, HBx down-regulated the expression of p21 and p27 then control. BCP1 mutant has more down-regulated the expression of p27 compared with HBx. In addition, expression of p53 was down-regulated by HBx and BCP1 mutant (Fig.2A and B). These results have indicated that HBx and BCP1 mutant down-regulate the negative cell cycle regulator such as p21, p27 and p53. Moreover, BCP1 mutant especially down-regulate the expression of p27 in transiently (data not shown) or stably HBx-expression Huh7 cells (Fig. 2B).

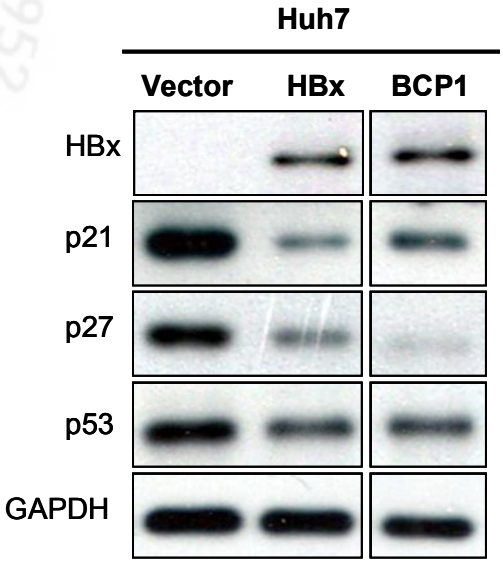
Down-regulation of cell cycle regulator protein may affect cell cycle. To determine the effect of HBx or BCP1 mutant on cell cycle progression in Huh7 cells. We has measured cell cycle by propidium iodide (PI) staining and flow cytometry (FACS) (Fig. 2C). In Huh7 cells

Collection @ jeju

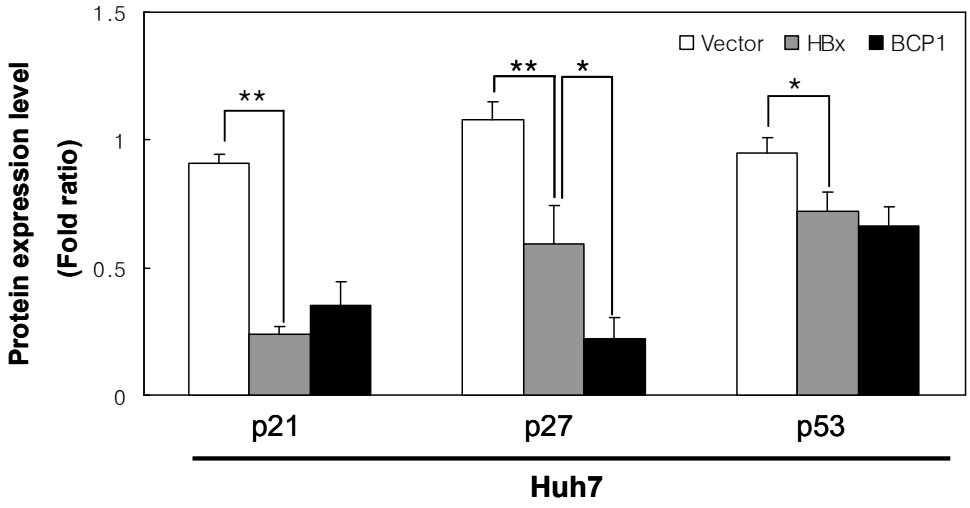
stably expressing HBx or BCP1, proportion of G0/G1 phase were decreased in BCP1 compared with HBx (53.74% for Huh7-HBx, 47.68% for Huh7-BCP1and increased the proliferation index (PI) (45.85% for Huh7-HBx, 52.87% for Huh7-BCP1 respectively, Fig. 2D).The proliferation index (PI) were calculated using the formula (PI=(G2M+S)/(G0/G1+S+G2M)*100). These results suggest that HBx enhance the proliferation in Huh7 cells. Moreover, BCP1 mutant promote the proliferation Huh7 cells more than HBx.

Collection @ jeju

A

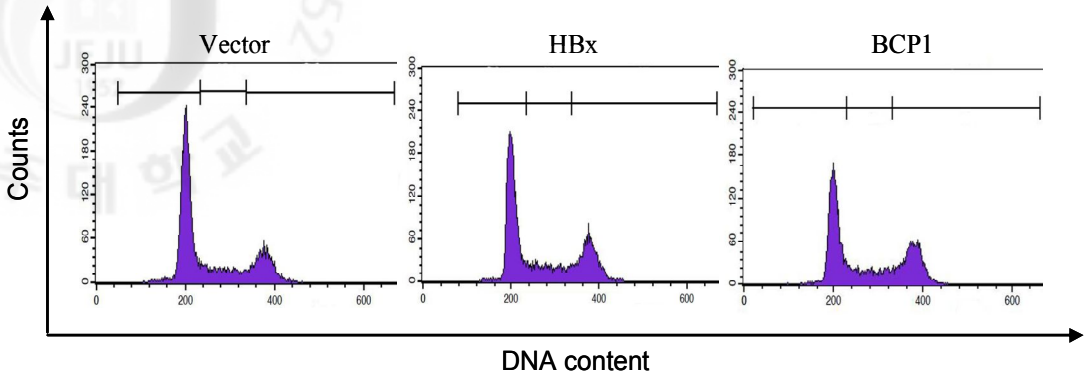


B

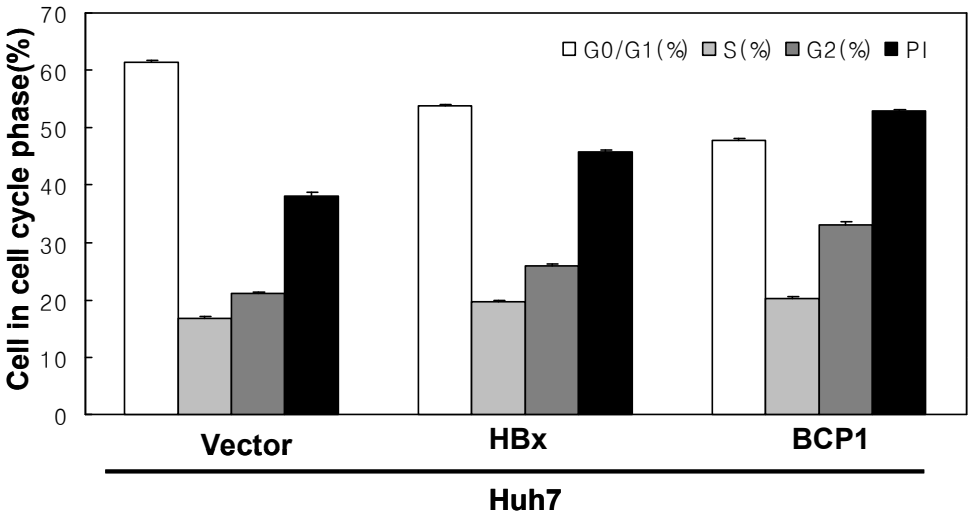


dCollection @ jeju

C



D



% Cell cycle Distribution

	Huh7		
	Vector	HBx	BCP1
G0/G1(%)	61.44±0.39	53.74±0.32	47.68±0.46
S(%)	16.81±0.34	19.54±0.26	20.16±0.36
G2(%)	21.14±0.84	25.96±0.12	33±0.69
PI	38.18±0.4	45.84±0.35	52.87±0.47

dCollection @ jeju

Figure 7. The effects of expression of HBx or BCP1 mutant on the regulation of cell cycle in Huh7 cells. Huh7 cells were transfected stably (B and E) with HBx and BCP1. The proteins were extracted by lysis buffer and analyzed by western blot. (A) The histogram shows the results from applying Image J program (B). Values shown represent the mean ± s.d. of three indepenent experiment. *P<0.05 and **P<0.01 versus control (Vector). Cell were stained with PI and cell cycle was examined by flowcytometry (C) and the cell-cycle was analyzed by BD software. Proliferation index (PI)=(G2M+S)/(G0G1+S+G2M)*100 was calculated (D).

Collection @ jeju

4.3. BCP1 mutant inhibits production of cleaved PARP as a marker for apoptosis protein in Huh7 cells.

The HBx was also reported to inhibit the apoptosis via up-regulation of anti-apoptotic proteins such as Bcl-2 and Survivin, inhibition of p21, p27 expression [3, 18, 24, 31] or activation of Notch signaling [26] .

To investigate effect of HBx or BCP1 mutant on the modulation of apoptosis in Huh7 cells, we examined the expression of apoptosis regulatory proteins by western blot. When comparing control vector and HBx, apoptotic proteins such as Bax and cleaved PARP or anti-apoptotic proteins such as Bcl-2 and Survivin expression did not change in Huh7 cells transiently (data not shown) or stably expressing HBx (Fig. 3A). However, BCP1 mutant decreased the expression of Bax and production of cleaved PARP than HBx in transiently transfected cells (data not shown). In stable cells, expression of Bax, Bcl-2 and survivin did not show meaningful change than Huh7-X cells, but, BCP1 mutant decreased the production of cleaved PARP dramatically (Fig. 3 A and B). These results have indicated that BCP1 mutant inhibits the apoptosis via decrease of production of cleaved PARP in Huh7 cells.

Collection @ jeju

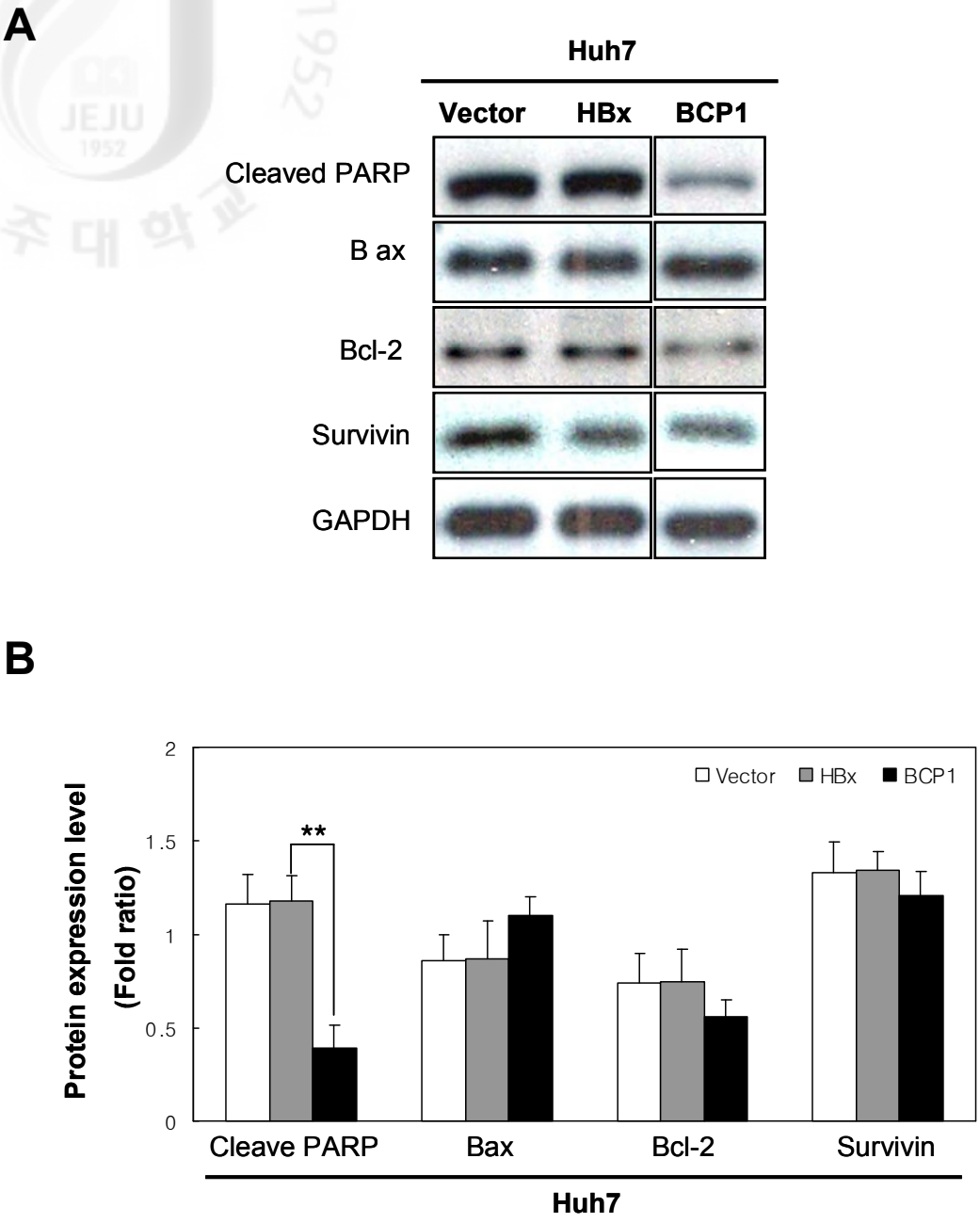


Figure 8. BCP1 mutant inhibit apoptosis in Huh7 cells. Huh7 cells were transfected stably with HBx and BCP1 mutant. Protein levels were analyzed by western blot using specific antibodies (A). Values shown represent the mean ± s.d. of three indepenent experiments. *P<0.05 and **P<0.01 versus control (HBx) (B).

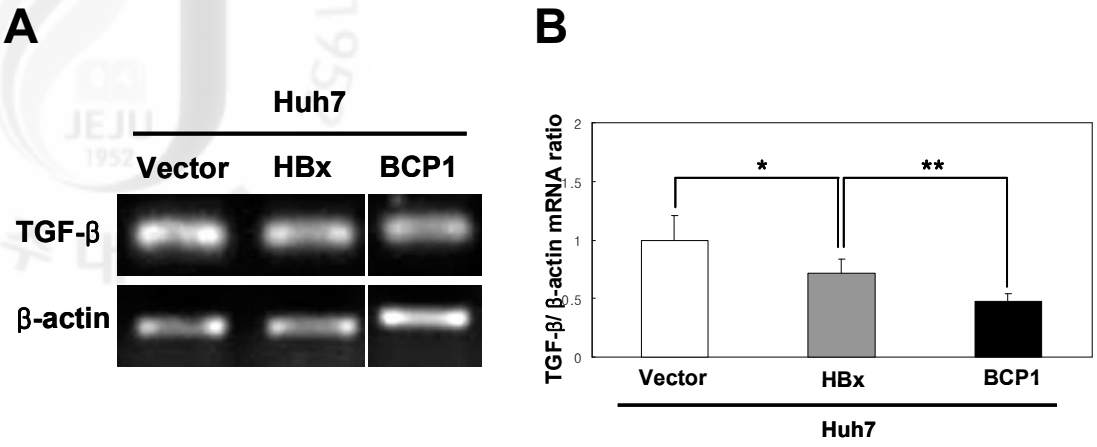
Collection @ jeju

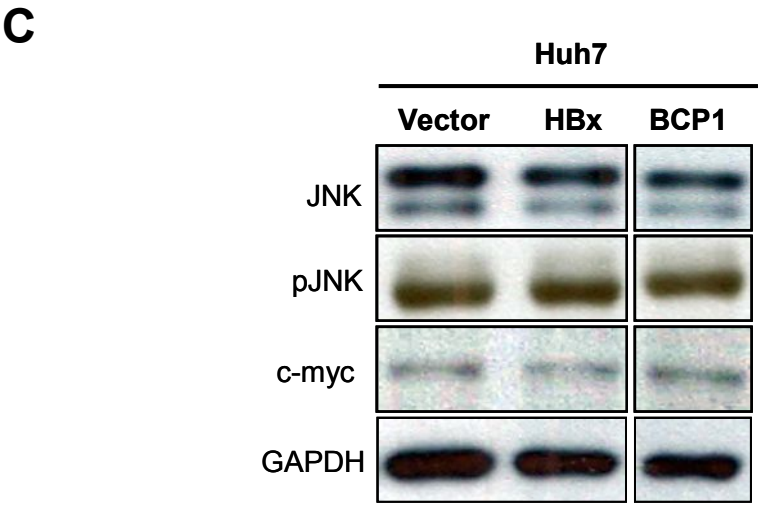
4.4. Huh7-BCP1 cells more down-regulated expression of TGF-β than Huh7-HBx cells, but, HBx or BCP mutant were not influenced in activated JNK dependent oncogenic pathways.

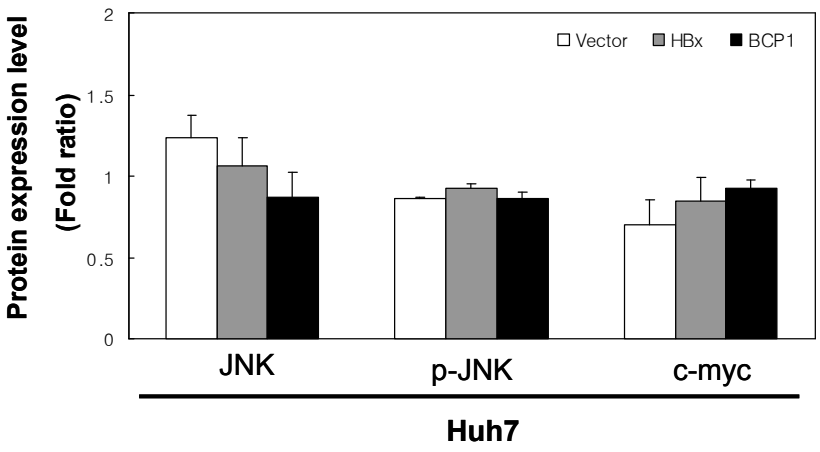
TGF-β is important regulatory suppressor for suppression of proliferation and cell death in liver cells [32]. To investigate the expression level of TGF-β in Huh7-HBX or Huh7-BCP1 cells, we isolated the mRNA in each cells and the expression level of TGF-β mRNA was analyzed by RT-PCR analysis. Expression of TGF-β was down-regulated in Huh7-HBx cells. Moreover, its level was more down-regulated by BCP1 in Huh7- BCP1 cells (Fig. 4 A and B).

HBx shifts TGF-β signaling from tumor-suppressive to JNK/pSmad3L/c-Myc oncogenic pathway in early carcinogenic process [33]. We analyzed expression of proteins of JNK/pSmad3L/c-Myc oncogenic pathway by western blot in Huh7-HBx or Huh-BCP1 cells. However, HBx or BCP1 mutant didn't affect the modulation of proteins of JNK/pSmad3L/c-Myc oncogenic pathway in Huh7-X or Huh7-BCP1 cells (Fig. 4 C and D).

Collection @ jeju







dCollection @ jeju

Figure 9. Expression levels of TGF-β and JNK-dependent oncogenic pathway proteins in Huh 7 cells expressing HBx or BCP1 mutant. Total RNA was isolated from each cells and cDNA synthesized as described in methods. PCR products of TGF-β using specific primers were analyzed by 1% agarose gel electrophoresis(A) and (B). JNK-dependent oncogenic proteins were analyzed by western blot (C and D). Values shown represent the mean ± s.d. of three indepenent experiments. *P<0.05 and **P<0.01 versus control (Huh7-Vector or Huh7-HBx)(C) Protein levels were analyzed by western blot analysis.

Collection @ jeju

6. Discussion

In the present study, we focus and studied intracellular roles of HBx mutant on the control of cell cycle and apoptosis in Huh7 cell lines. Our results have demonstrated that HBx mutant (BCP1) more significantly influenced the modulation of cell cycle progression and apoptosis than HBx in Huh7 cells.

The mutation of HBV basal core promoter (BCP) is associated with hepatocelluar carcinoma (HCC) [1, 6-8] and BCP is overlapped with C-terminal region of the hepatitis B virus x (HBx, nt 1742-1835) protein [3, 9] (Fig. 1 A). BCP mutant enhance the HBV replication and increase the incidence of hepatocelluar carcinoma (HCC) [9]. Moreover, BCP 1753 mutant (BCP1, A1762T and G1764A, T1753C nucleotide exchange) more increase the occurrence of HCC than the common BCP double mutation (A1762T and G1764A nucleotide exchange) [9]. In this study, we used the clinical HBx mutant (BCP1; T1753C, A1762T and G1764A) and its region of mutation has indicated the box of Figure 1 A.

Our clinical HBx mutant (BCP1) and wild type HBx localized in cytosol and mitochodria. However, BCP1 mutant did not affect the intracellular localization compared with HBx (Fig. 1 B). C-terminus of HBx is necessary region for the specific localization of mitochondria [18,

Collection @ jeju

29] and mitochondria localization of HBx has been affected various intracellular environment [16, 29, 34, 35]. Mutation of 115th amino acid at the C-terminus of HBx was distributed in the cytoplasm without specific organelles of intracellular [29]. Even BCP1 mutations are located in C-terminus region, they did not alter the location of proteins.

The deletion of C-terminal of HBx was frequently found in HBV-associated HCC tissues. Then the deletion of 20 and 40 amino acids at the COOH-terminus, cell cycle progression and tumorigenesis were promoted in Huh7 cells [36]. A natural mutant of HBx with a deletion from 382 to 401 base pairs at the C-terminal (HBxΔ127) also promoted cell growth [27, 28, 37]. Interestingly, the deletion sequence in natural C-terminal deletion mutant (HBxΔ127) exactly overlapped our mutation amino acid site 127, 130, 131. Wang et al explained HBxΔ127 mutant promote hepatoma cell growth through activation SREBP-1c involving fatty acid synthase (FAS) [37]. Two separate approaches showed that the ablation of FAS activity causes a dramatic down-regulation of S-phase kinase-associated protein 2 (Skp2), a component of the E3 ubiquitin ligase that controls the turnover of $p27^{Kip1}$ and p21, and ultimately tie into the retinoblastoma protein pathway and lead to a cell-cycle arrest [38]. We believe that BCP1 mutation of HBx increase binding affinity of SREBP-1c and activate FAS, which activate the Skp2 and promote the proteasomal degradation of $p27^{Kip1}$ as in HBxΔ127. Skp2 is also involved in degradation of p21. In HepG2 cells, HBx mutant with

Collection @ jeju

changes in the BCP region decreased levels of p21 and increased cyclin E expression [30]. These findings have showed possibility that BCP1 may increase the occurrence of HCC via promotion of cell cycle progression than HBx.

HBx inhibit the induction of apoptosis by inhibition of anti-apoptotic proteins such as Bcl-2 and Survivin [3, 18, 24, 31] and HBV X protein truncated 27 amino acids at the COOH terminal up-regulated transcriptional activity of survivin and failed to induce apopoptosis [28]. However, exact role of our clinical HBx mutant (BCP 1753: BCP1) on the modulation of apoptosis has not been enough examined. Interestingly, BCP1 mutant interrupted the apoptosis through inhibition of cleaved PARP production in Huh7-BCP1 cells (Fig. 3). These results provide another clue at increase of occurrence of HCC by BCP1 mutant.

TGF-β controls the cyclin-dependent kinase inhibitor such as p15, p21 and p27. In addition, TGF-β induce the G1 phase arrest via $p27^{kip1}$ [39] and prevents proteasomal degradation of p27 for cell cycle arrest [40]. Cell death by TGF-β in liver cells requires the production of reactive oxygen species (ROS) through increase of NOX4 expression. The ROS production cause the loss of mitochondrial transmembrane potential, an increase of cytochrome c release and activation of caspase [8, 12]. Interestingly, in our results have indicated that HBx and BCP1 mutant down-regulate the mRNA expression of TGF-β, moreover, BCP1 mutant more decrease the expression of TGF-β compared with HBx (Figure

Collection @ jeju

4). These findings suggest that BCP mutant may control the promotion of cell cycle progression and inhibition of apoptosis more than HBx via high reducing effect of expression of TGF-β in Huh7 cells.

It has been reported that HBx shifts TGF-β signaling from tumor-suppressive pathway to oncogenic pathway in early carcinogenic process of HBV-infected hepatocyte [33]. In this study, HBx or BCP1 mutant didn't more affected modulation of activated JNK-dependent oncogenic pathway proteins (Fig. 4 C). These results were considered that modulation of TGF-β by HBx or BCP1 mutant did not influence the activated-JNK-dependent oncogenic pathway in already oncogenic signals activated-hepatoma cell.

In summary, our study provides a possible mechanism by which HBV core promoter mutations are associated with an increased risk of HCC. The results suggest that core promoter mutations seen in patients with HCC promote carcinogenesis possibly via down regulation of p27 expression and in turn acceleration of cell cycle progression, and cellular proliferation and inhibition of apoptosis.

Collection @ jeju

Ⅲ. REFERENCES

1. PART Ⅰ

1 Yin J, Xie J, Liu S *et al.* Association between the various mutations in viral core promoter region to different stages of hepatitis B, ranging of asymptomatic carrier state to hepatocellular carcinoma. *Am J Gastroenterol* 2010; **106:** 81-92.

2 Tang H, Oishi N, Kaneko S *et al.* Molecular functions and biological roles of hepatitis B virus x protein. *Cancer science* 2006; **97:** 977-983.

3 Kim KH. Pro-apoptotic function of hepatitis B virus X protein. *The Korean Journal of Hepatology* 2010; **16:** 112-122.

4 Ye L, Dong N, Wang Q *et al.* Progressive changes in hepatoma cells stably transfected with hepatitis B virus X gene. *Intervirology* 2008; **51:** 50-58.

5 Shih WL, Kuo ML, Chuang SE *et al.* Hepatitis B virus X protein activates a survival signaling by linking SRC to phosphatidylinositol 3-kinase. *J Biol Chem* 2003; **278:** 31807.

6 Hsieh A, Kim HS, Lim SO *et al.* Hepatitis B viral X protein interacts with tumor suppressor adenomatous polyposis coli to activate Wnt/[beta]-catenin signaling. *Cancer Lett* 2011; **300:** 162-172.

7 Bui-Nguyen T, Pakala S, Sirigiri R *et al.* NF-κB signaling mediates the induction of MTA1 by hepatitis B virus transactivator protein HBx. *Oncogene* 2009; **29:** 1179-1189.

8 Neuveut C, Wei Y, Buendia MA. Mechanisms of HBV-related hepatocarcinogenesis. *J Hepatol* 2010; **52:** 594-604.

9 Park NH, Chung YH. Molecular mechanisms of hepatitis B virus-associated hepatocellular carcinoma. *The Korean Journal of Hepatology* 2007; **13:** 320-340.

dCollection @ jeju

10 Kuo CY, Wu CC, Hsu SL *et al.* HBx Inhibits the Growth of CCL13-HBX-Stable Cells via the GSK-3/-Catenin Cascade. *Intervirology* 2008; **51:** 130-136.

11 Kuo CY, Wang JC, Wu CC *et al.* Effects of hepatitis B virus X protein (HBx) on cell-growth inhibition in a CCL13-HBx stable cell line. *Intervirology* 2008; **51:** 26-32.

12 Kuo CY, Wang JC, Hsu SL *et al.* Functional Characterization of Hepatitis B Virus X Protein Based on the Inhibition of Tumorigenesis in Nude Mice Injected with CCL13-HBx Cells. *Intervirology* 2008; **51:** 253-260.

13 Wang JC, Hsu SL, Hwang GY. Inhibition of tumorigenicity of the hepatitis B virus X gene in Chang liver cell line. *Virus Res* 2004; **102:** 133-139.

14 Mukherji A, Janbandhu VC, Kumar V. HBx-dependent cell cycle deregulation involves interaction with cyclin E/A–cdk2 complex and destabilization of p27Kip1. *Biochem J* 2007; **401:** 247.

15 Park SG, Chung C, Kang H *et al.* Up-regulation of cyclin D1 by HBx is mediated by NF-κB2/BCL3 complex through κB site of cyclin D1 promoter. *J Biol Chem* 2006; **281:** 31770-31777.

16 Jung JK, Arora P, Pagano JS *et al.* Expression of DNA methyltransferase 1 is activated by hepatitis B virus X protein via a regulatory circuit involving the p16INK4a-cyclin D1-CDK 4/6-pRb-E2F1 pathway. *Cancer Res* 2007; **67:** 5771.

17 Zhang W, Xu F, Shan C *et al.* Gene expression profiles of human liver cells mediated by hepatitis B virus X protein. *Acta Pharmacol Sin* 2009; **30:** 424-434.

18 Zhang X, Dong N, Yin L *et al.* Hepatitis B virus X protein upregulates survivin expression in hepatoma tissues. *J Med Virol* 2005; **77:** 374-381.

19 Martin‐Vilchez S, Lara‐Pezzi E, Trapero‐Marugán M *et al.* The molecular and pathophysiological implications of hepatitis BX antigen in chronic hepatitis B virus infection. *Rev Med Virol* 2011; .

20 Nagata H, Hatano E, Tada M *et al.* Inhibition of c‐Jun NH2‐terminal kinase switches Smad3 signaling from oncogenesis to tumor‐suppression in rat hepatocellular carcinoma. *Hepatology* 2009; **49:** 1944-1953.

Collection @ jeju

21 Sekimoto G, Matsuzaki K, Yoshida K *et al.* Reversible Smad-dependent signaling between tumor suppression and oncogenesis. *Cancer Res* 2007; **67:** 5090.

22 Kretzschmar M, Doody J, Timokhina I *et al.* A mechanism of repression of TGFβ/Smad signaling by oncogenic Ras. *Genes Dev* 1999; **13:** 804.

23 Murata M, Matsuzaki K, Yoshida K *et al.* Hepatitis B virus X protein shifts human hepatic transforming growth factor (TGF) - β signaling from tumor suppression to oncogenesis in early chronic hepatitis B. *Hepatology* 2009; **49:** 1203-1217.

24 Gearhart TL, Bouchard MJ. The hepatitis B virus HBx protein modulates cell cycle regulatory proteins in cultured primary human hepatocytes. *Virus Res* 2011; **155:** 363-367.

25 Wang T, Zhao R, Wu Y *et al.* Hepatitis B virus induces G1 phase arrest by regulating cell cycle genes in HepG2. 2.15 cells. *Virology Journal* 2011; **8:** 231.

26 Albright CD, Salganik RI, Craciunescu CN *et al.* Mitochondrial and microsomal derived reactive oxygen species mediate apoptosis induced by transforming growth factor - β1 in immortalized rat hepatocytes. *J Cell Biochem* 2003; **89:** 254-261.

27 Toyoshima H, Hunter T. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell* 1994; **78:** 67-74.

28 Wei Y, Liu D, Zhou F *et al.* Identification of β-1, 4-galactosyltransferase I as a target gene of HBx-induced cell cycle progression of hepatoma cell. *J Hepatol* 2008; **49:** 1029-1037.

29 Oshikawa O, Tamura S, Kawata S *et al.* The Effect of Hepatitis B Virus X Gene Expression on Response to Growth Inhibition by Transforming Growth Factor-[beta] 1. *Biochem Biophys Res Commun* 1996; **222:** 770-773.

30 Zhang YE. Non-Smad pathways in TGF-β signaling. *Cell Res* 2008; **19:** 128-139.

31 Carmona - Cuenca I, Herrera B, Ventura JJ *et al.* EGF blocks NADPH oxidase activation by TGF - β in fetal rat hepatocytes, impairing oxidative stress, and cell death. *J Cell Physiol* 2006; **207:** 322-330.

Collection @ jeju

32 Caja L, Sancho P, Bertran E *et al.* Overactivation of the MEK/ERK Pathway in Liver Tumor Cells Confers Resistance to TGF-β–Induced Cell Death through Impairing Up-regulation of the NADPH Oxidase NOX4. *Cancer Res* 2009; **69:** 7595.

33 Chan DW, Ng O. Knock‐down of hepatitis B virus X protein reduces the tumorigenicity of hepatocellular carcinoma cells. *J Pathol* 2006; **208:** 372-380.

2. PART Ⅱ

1 Yin J, Xie J, Liu S *et al.* Association between the various mutations in viral core promoter region to different stages of hepatitis B, ranging of asymptomatic carrier state to hepatocellular carcinoma. *Am J Gastroenterol* 2010; **106:** 81-92.

2 Neuveut C, Wei Y, Buendia MA. Mechanisms of HBV-related hepatocarcinogenesis. *J Hepatol* 2010; **52:** 594-604.

3 Park NH, Chung YH. Molecular mechanisms of hepatitis B virus-associated hepatocellular carcinoma. *The Korean Journal of Hepatology* 2007; **13:** 320-340.

4 Tang H, Oishi N, Kaneko S *et al.* Molecular functions and biological roles of hepatitis B virus x protein. *Cancer science* 2006; **97:** 977-983.

5 Tong MJ, Blatt LM, Kao JH *et al.* Basal core promoter T1762/A1764 and precore A1896 gene mutations in hepatitis B surface antigen‐positive hepatocellular carcinoma: a comparison with chronic carriers. *Liver International* 2007; **27:** 1356-1363.

6 Tanaka Y, Mukaide M, Orito E *et al.* Specific mutations in enhancer II/core promoter of hepatitis B virus subgenotypes C1/C2 increase the risk of hepatocellular carcinoma. *J Hepatol* 2006; **45:** 646-653.

7 Liu S, Xie J, Yin J *et al.* A matched case–control study of hepatitis B virus mutations in the preS and core promoter regions associated independently with hepatocellular carcinoma. *J Med Virol* 2011; **83:** 45-53.

Collection @ jeju

8 Tong MJ, Blatt LM, Kao JH *et al.* Precore/basal core promoter mutants and hepatitis B viral DNA levels as predictors for liver deaths and hepatocellular carcinoma. *World Journal of Gastroenterology* 2006; **12:** 6620.

9 Yim HJ. Hepatitis B virus genetic diversity and mutant. *The Korean Journal of Hepatology* 2008; **14:** 446-464.

10 Jammeh S, Tavner F, Watson R *et al.* Effect of basal core promoter and pre-core mutations on hepatitis B virus replication. *J Gen Virol* 2008; **89:** 901.

11 Hussain Z, Jung HS, Ryu DK *et al.* Genetic dissection of naturally occurring basal core promoter mutations of hepatitis B virus reveals a silent phenotype in the overlapping X gene. *J Gen Virol* 2009; **90:** 2272.

12 Tacke F, Gehrke C, Luedde T *et al.* Basal core promoter and precore mutations in the hepatitis B virus genome enhance replication efficacy of lamivudine-resistant mutants. *J Virol* 2004; **78:** 8524.

13 Chu CJ, Keeffe EB, Han SH *et al.* Prevalence of HBV precore/core promoter variants in the United States. *Hepatology* 2003; **38:** 619-628.

14 Fang ZL, Sabin CA, Dong BQ *et al.* The association of HBV core promoter double mutations (A1762T and G1764A) with viral load differs between HBeAg positive and anti-HBe positive individuals: a longitudinal analysis. *J Hepatol* 2009; **50:** 273-280.

15 Chun YK, Youn Kim J, Jung Woo H *et al.* No significant correlation exists between core promoter mutations, viral replication, and liver damage in chronic hepatitis B infection. *Hepatology* 2000; **32:** 1154-1162.

16 Ma J, Sun T, Park S *et al.* The role of hepatitis B virus X protein is related to its differential intracellular localization. *Acta biochimica et biophysica Sinica* 2011; **43:** 583-588.

17 Kim KH. Pro-apoptotic function of hepatitis B virus X protein. *The Korean Journal of Hepatology* 2010; **16:** 112-122.

18 Ye L, Dong N, Wang Q *et al.* Progressive changes in hepatoma cells stably transfected with hepatitis B virus X gene. *Intervirology* 2008; **51:** 50-58.

dCollection @ jeju

19 Mukherji A, Janbandhu VC, Kumar V. HBx-dependent cell cycle deregulation involves interaction with cyclin E/A–cdk2 complex and destabilization of p27Kip1. *Biochem J* 2007; **401:** 247.

20 Park SG, Chung C, Kang H *et al.* Up-regulation of cyclin D1 by HBx is mediated by NF-κB2/BCL3 complex through κB site of cyclin D1 promoter. *J Biol Chem* 2006; **281:** 31770-31777.

21 Jung JK, Arora P, Pagano JS *et al.* Expression of DNA methyltransferase 1 is activated by hepatitis B virus X protein via a regulatory circuit involving the p16INK4a-cyclin D1-CDK 4/6-pRb-E2F1 pathway. *Cancer Res* 2007; **67:** 5771.

22 Zhang W, Cai N, Ye L *et al.* Transformation of human liver L-O2 cells mediated by stable HBx transfection. *Acta Pharmacol Sin* 2009; **30:** 1153-1161.

23 Wei Y, Liu D, Zhou F *et al.* Identification of β-1, 4-galactosyltransferase I as a target gene of HBx-induced cell cycle progression of hepatoma cell. *J Hepatol* 2008; **49:** 1029-1037.

24 Zhang X, Dong N, Yin L *et al.* Hepatitis B virus X protein upregulates survivin expression in hepatoma tissues. *J Med Virol* 2005; **77:** 374-381.

25 Iyer S, Groopman JD. Interaction of mutant hepatitis BX protein with p53 tumor suppressor protein affects both transcription and cell survival. *Mol Carcinog* 2011; .

26 Wang F, Zhou H, Xia X *et al.* Activated Notch signaling is required for hepatitis B virus X protein to promote proliferation and survival of human hepatic cells. *Cancer Lett* 2010; **298:** 64-73.

27 Wang Q, Zhang W, Ye L *et al.* A mutant of HBx (HBxΔ127) promotes hepatoma cell growth via sterol regulatory element binding protein 1c involving 5-lipoxygenase. *Acta Pharmacol Sin* 2010; **31:** 367-374.

28 Zhang H, SHAN C, Li N *et al.* Identification of a natural mutant of HBV X protein truncated 27 amino acids at the COOH terminal and its effect on liver cell proliferation1. *Acta Pharmacol Sin* 2008; **29:** 473-480.

Collection @ jeju

29 Li SK, Ho SF, Tsui KW *et al.* Identification of functionally important amino acid residues in the mitochondria targeting sequence of hepatitis B virus X protein. *Virology* 2008; **381:** 81-88.

30 Huang Y, Tong S, Tai AW *et al.* Hepatitis B Virus Core Promoter Mutations Contribute to Hepatocarcinogenesis by Deregulating SKP2 and Its Target, p21. *Gastroenterology* 2011; .

31 Zhang W, Xu F, Shan C *et al.* Gene expression profiles of human liver cells mediated by hepatitis B virus X protein. *Acta Pharmacol Sin* 2009; **30:** 424-434.

32 Caja L, Sancho P, Bertran E *et al.* Overactivation of the MEK/ERK Pathway in Liver Tumor Cells Confers Resistance to TGF-β–Induced Cell Death through Impairing Up-regulation of the NADPH Oxidase NOX4. *Cancer Res* 2009; **69:** 7595.

33 Murata M, Matsuzaki K, Yoshida K *et al.* Hepatitis B virus X protein shifts human hepatic transforming growth factor (TGF) - β signaling from tumor suppression to oncogenesis in early chronic hepatitis B. *Hepatology* 2009; **49:** 1203-1217.

34 Clippinger AJ, Gearhart TL, Bouchard MJ. Hepatitis B Virus X Protein Modulates Apoptosis in Primary Rat Hepatocytes by Regulating both NF-{kappa} B and the Mitochondrial Permeability Transition Pore. *J Virol* 2009; **83:** 4718.

35 Clippinger AJ, Bouchard MJ. Hepatitis B virus HBx protein localizes to mitochondria in primary rat hepatocytes and modulates mitochondrial membrane potential. *J Virol* 2008; **82:** 6798.

36 Liu X, Zhang S, Lin J *et al.* Hepatitis B virus X protein mutants exhibit distinct biological activities in hepatoma Huh7 cells. *Biochem Biophys Res Commun* 2008; **373:** 643-647.

37 Wang Q, Zhang W, Liu Q *et al.* A Mutant of Hepatitis B Virus X Protein (HBxΔ127) Promotes Cell Growth through A Positive Feedback Loop Involving 5-Lipoxygenase and Fatty Acid Synthase. *Neoplasia (New York, NY)* 2010; **12:** 103.

38 Knowles LM, Axelrod F, Browne CD *et al.* A fatty acid synthase blockade induces tumor cell-cycle arrest by down-regulating Skp2. *J Biol Chem* 2004; **279:** 30540.

dCollection @ jeju

39 Toyoshima H, Hunter T. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell* 1994; **78:** 67-74.

40 Lecanda J, GONAPATHY V, D'AQUINO-ARDALAN C *et al.* TGFβ prevents proteasomal degradation of the cyclin-dependent kinase inhibitor p27kip1 for cell cycle arrest. *Cell cycle* 2009; **8:** 742-756.

dCollection @ jeju

Ⅳ. 요약문

1. PART Ⅰ

HBx 는 B 형간염바이러스(HBV) 감염에 필수적이며, HBV 증식, HBV 감염에 의한 간질환 및 간암(HCC)의 발달에 영향을 미친다. 그러나 HBx 의 정확한 역할은 아직 논란이 되고 있다. 본 연구에서, 우리는 논란이 되고 있는 HBx 의 역할에 대해 일반 간세포와 간암세포주에서 HBx 에 의한 세포주기와 세포자멸사 조절에 초점을 맞추고 실험을 하였다.

우리는 본 연구를 위해 HBx 가 지속적으로 발현하는 Huh7-X 와 Chang-X 세포주를 만들었으며, HBx 단백질은 각 세포에서 세포주기와 p27, TGF-β 발현을 다르게 조절 하는 것을 보여주었다. 더욱이, HBx 는 Chang-X 세포에서 특이적으로 Bcl-2 의 발현을 증가시켰으며, Cleaved PARP 단백질의 형성을 감소시켰다. 그리고 HBx 는 Chang 과 Huh7 세포에서 serum starvation 과 같은 세포죽음을 발생할 수 있는 조건에서 세포자멸사를 억제시켰다. 이러한 결과는, HBx 가 간암세포와 일반 간세포에서 다르게 세포주기와 세포자멸사를 조절 하는 것을 나타냈으며, 이는 논란이 되고 있는 HBx 의 세포내 역할에 대한 단서를 제공 하였다.

dCollection @ jeju

2. PART Ⅱ

Hepatocellular carcinoma (HCC)는 일반적으로 발생빈도가 높은 암 중의 하나이며, HCC 와 HCC와 관련된 질병은 B형 간염바이러스(HBV)가 원인이 된다. HBV 는 basal core promoter (BCP) 지역에 종종 변이가 발생 하며, 이 지역은 HBx의 open reading frame (ORF)과 겹쳐져 발생한다. 또한 HBx 는 간암 발생에 있어 중요한 역할을 한다. 본 연구에서는 Huh-7 간암세포에서의 HBx 변이(BCP1) 의 영향에 대하여 조사를 하였다. 우리는 clinical samples 로 부터 HBx의 변이를 클로닝 하였으며, 이들을 Huh-7 세포에 일시적 또는 안정적으로 transfection 시켜 실험을 하였다. HBx 변이체(BCP1) 는 HBx 에 비해 특이적으로 CDK inhibitor p27kip1 의 발현을 감소 시켰으며, 세포주기를 더욱 촉진 시켰다. 또한 BCP1 은 HBx 에 비해 cleaved PARP 의 발생을 억제시켰으며, multifunctional cytokine인 TGF-β는 발현을 더욱 감소 시켰다. 따라서 BCP1 변이체는 HBx에 비해 Huh-7 세포에서 더욱 세포주기를 촉진시키며 세포자멸사를 억제하였으며, 이는 아마 TGF-β 의 발현 감소를 필요로 할 것이라는 것을 나타내었다.

Collection @ jeju

감사의글

대학을 졸업하는 과정에서 취업과 공부의 두 가지 갈림길을 놓고 많은 고민끝에 대학원 입학을 결정을 내리고 석사 과정을 시작한지 어느덧 2 년이라는 시간이 지나갔습니다.

2 년여의 시간동안 많은 일들이 있었고, 그 과정에서 많은 배움 들이 있었습니다. 처음 입학 후 논문 보는 법을 몰라 해매기도 하고, 처음 접해보는 실험들과 저에게 쏟아져 밀려오는 많은 지식들 속에서 나의 한계를 느꼈고, 대학원 1 학기를 잘 적응 하지 못하고 많은 방황을 하였습니다.

하지만 그러한 방황 속에서 다시 제자리를 찾고 공부할 수 있도록 배려해 주신 조문제 교수님께 먼저 감사의 말씀을 드립니다. 또한 실험적, 이론적 지식뿐만 아니라 사회생활에 필요한 많은 조언을 해주시고 언제든지 찾아와도 좋다고 말씀해주셔서 감사드립니다. 평생 모시고 찾아 뵐 수 있는 선생님이 한 분 더 생겼다는 것이 저에게는 이번 석사를 졸업하면서 얻고 가는 가장 좋은 선물 중 하나 인 것 같습니다.

그리고 학부 전공과는 다른 대학원에 입학했음에도 불구하고 언제나 챙겨주시고, 찾아 뵐 때 마다 항상 기쁘게 맞아주시는 하진환 교수님께도 깊은 감사의 말씀을 드립니다. 그리고 지금은 다른 곳으로 자리를 옮기셨지만 제가

dCollection @ jeju

의과대학 대학원에 입학을 할 수 있도록 인도 해주시고, 처음 실험실에 들어와 실험에 대한 기초 지식과 이론적 지식에 대한 많은 조언을 아끼지 않으시고 가르쳐 주신 남제국 선생님께도 감사를 드립니다. 무엇보다 실험실 생활이 힘들때 마다 옆에서 많은 힘이 되어주시고 격려해 주시고 많은 조언을 해주신 김영미 선생님께도 감사의 말씀을 드리고, 이번에 박사학위 받으신 것 진심으로 축하 드립니다.

그리고 남자들이 비교적 적은 실험실에서 친형같이 함께 해주시고 많은 도움을 주신 기천이형과 동생같이 아껴주신 멋지신 상철이형에게도 고맙다는 말씀을 드리고 싶습니다. 마지막으로 항상 저의 든든한 버팀목이 되어주시고 어떤길을 선택해도 지지해주시는 부모님께 감사를 드리고, 무엇보다 모든 것을 이끌어 주신 하나님께 감사를 드립니다.

옆에서 도와주시고 함께 해주신 많은 분들께 다시한번 감사의 말씀을 드립니다.

감사합니다.

2012 년 1 월

양 창 희

dCollection @ jeju