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THE BIOLOGICAL EFFECT OF B-SITOSTEROL ON HEPATOMA CELL HEPG2 TRANSFECTED WITH HBX GENE

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ABSTRACT

Hepatitis B virus (HBV) infection is endemic disease in many areas around the world and is considered as one of the risk factor for hepatocellular carcinoma .The latter is the fifth leading cause of death and rapidly increased and became a global health problem. There is an outstanding and urgent need for the development of effective treatment for chronic hepatitis and HCC. The aim of this study was to impair the hepatitis b virus replication and cancer metastasis. To explore this issue we investigated the effect of β-Sitosterol on biological process of hepatoma cell line. HepG2 transfected with hepatitis B x gene. The cell migration and gene expression were studied using gene chip. Gene Ontology result shows that β-Sitosterol has an effect on HepG2-HBx. The Gene Set Enrichment Analysis shows the impact of β-Sitosterol is negative regulation of megakaryocyte, telomere maintenance and extrinsic apoptotic signaling pathway. This result may open new areas of hepatitis B research.

KEYWORDS: HCC, HBV, gene expression, cell migration megakaryocyte, telomere maintenance.

INTRODUCTION

Epidemiological and experimental studies have suggested a protective role of beta-sitosterol in development of some types of cancers, such as breast, colon and prostate cancer. [1, 2, 3 and 4], and it's suppression of expression of the genes involved in cell proliferation reported of Hela cells.[5] In this study, we investigate the effect of β-Sitosterol on hepatoma cell line transfected with HBx and its effect on hepatocellular carcinoma. Hepatocellular carcinoma (HCC) reported as the fifth most common cancer in men and eighth in women and rapidly increases in the world.[6, 7] The disease is mainly associated with chronic hepatitis B virus (HBV) infection.[8, 9, 10 and 15] Hepatitis B is highly endemic in sub-Sahara and South-East Asia,[11, 12], several viral proteins, including hepatitis Bx protein, truncated pres2/S protein and hepatitis B spliced protein (HBSP) were shown to be able to affect the cellular growth and apoptosis [13], The 154-amino-acid viral gene product 'X' is named X due to uncertainty about its function, the viral function that is probably implicated in oncogenesis. It's shown to be important for HBV replication.[14] The HBx mediates many cellular functions, including encoding of cytoskeleton and cell adhesion molecule, cell-cycle regulation, and tumor suppressor genes.[16, 17] Although it has been evident that the HBV whole-X protein is involved in the development of hepatocellular carcinoma, its biological role and molecular mechanism remain unknown.[18] HBx protein inactivates negative growth regulators, and inhibits the expression of p53 tumor suppressor gene as well as tumor suppressor genes and senescence-related factors. HBx protein causing hypermethylation of leading to silencing of tumor suppressor genes, or global hypomethylation that causing in chromosomal instability[19], The gene expression showed that HBx can lead to expression of molecules involved in signal transduction, transcriptional regulation, protein degradation, cell cycle control, apoptosis, metastasis, immune response and metabolism in mouse hepatocyte cells[20] in vitro we explore the effect of beta sitosterol on HepG2HBx and it could focus on new area of HBV pathogenesis.

MATERIALS AND METHOD

β-Sitosterol (**$C_{29}H_{50}O$**) (Sigma Aldrich analytical standard) prepared by dissolved 0.995mg β-Sitosterol in 10 ml Ethanol

HepG2.2.15 cells (CRL-11997; ATCC) harboring four copies of HBV-DNA.

HepG2 from American Type Culture Collection (ATCC).

Cell counting kit 8 CCK8, DOjinDO laboratories, Japan.

Cell culture: HepG2.2.15and HepG2 were cultured in Dulbecco's Modified Eagle Medium (Invitrogen) with 10% fetal bovine serum (FBS) and G418 Geneticin (Invitrogen) at 380 µg/ml.

Cell viability assay: Cell counting kit 8 (CCK8, DOjinDO laboratories, Japan) was used for the cell viability determination. $8x10^3$ cells from HepG 2 and HepG2.2.15 were counted (Count star Biotech Automated Cell Counter Shanghai) and cultured in 100 µl Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal bovine serum (FBS) in 96 wells plate for 24 hours, β-Sitosterol added in different concentration from 0.0, 60, 120, 240 µM . Incubated for 24 hours to determine the cell viability in selected time. One hour before measuring the viability of the cells, the Dulbecco's Modified Eagle Medium were changed with 100 µl of 1:10 CCK-8 solution and DMEM and incubated for one hour at 37°C in 5% CO2 incubator. Then we measured the absorbance at 450 nm using a microplate reader (ELx800 Absorbance Reader). Care was taken not to introduce bubbles to the wells during changing the medium, since they interfere with the O.D. reading.

Cell migration: (Transwell method)

HepG2.2.15 cells were pretreated with the desired concentration of β-Sitosterol (chemoattractent) for 24 hours .a 24wells format plate (costar, with 8µm pore size placed. Then upper layer of a cell permeable membrane were treated with collagen for 20 minutes. 8×10^4 HepG 2.2.15 cells were suspended in 500 µl DMEM without FBS were seeded in the upper layer of a cell permeable membrane. and 800 µl of DMEM with 10% FBS added to lower chamber and Incubated the cells in the Transwell plate at 37 °C and 5%CO2 for 24 hours. This allowed the cells to migrate toward the underside of the insert filter. We removed the cells that did not migrate through the pores by gently swabbed out with cotton swab from the upper side of the filter membrane with cotton swab in order to remove the cell debris. Then we stain the cells on the lower side of the insert filter with500ul 1%crystal violet (Beyotime.China) for five minutes. Removed excess crystal violet from the side by using the swab cotton. The cells were counted on the lower side of the filter under microscope. The same experiment procedure performed for control without chemo attractant.

Plasmid construction: Plasmids included HBx cDNA, were constructed and used as templates for polymerase chain reaction (PCR) amplification. PCR-generated HBx genes were inserted into the KpnI and XhoI sites of the pcDNA3.1/Hygro (+) plasmid (V870–20,

Invitrogen), the resultant vectors were called pcDNAhygro^{+}- HBVx. The primers used for PCR amplification is shown as followed

HBx-hygro (+) cloneF: 5' - CGGGGTACC GCC ACC ATGGCTGCTAGGCTG TGC -3'

HBx-hygro (+) cloneR: 5' - CCGCTCGAG TTA CTTGTCG TCATCGTCTT TGTAGTCGGC AGAGGTGAAAAAG -3'

Transfection: HepG2cell line were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Fetal bovine serum) in 10 cm plate till a 90% confluence reached then the 2ml Trypsin (Trypsin 0.25%EDTA (1x) gilbco) were added for 3minutes and removed 3ml of DMEM were added, HepG2 cells were counted and seeded in 6-wells plate at the density of 5× $\mathbf{10^5}$ per well 24 hours before transfection. The cells would a 90% confluence and the medium changed to DMEM without FBS and incubated for one hour before transfection. Plasmids were transected into HepG2 cells using lipofectamine 3000 (Invitrogen, Carlsbad, CA) prepared in two test tube as in tables I and 2. 24 hours after transfection the Hygromycin were added. The cells were monitored by changing the medium with the addition of Hygromycin 800µg/ml till reached the clear clone, and then we picked into 24 wells plate after the cells a 90% confluence, cells were passage into 12 wells plate, 6 wells plate, 6 cm plate, and then propagated in 10cm plate.

Table-1 Tube one shows the plasmid preparation

Component	Amount
pcDNAhygro+- HBVx	3.44 µl
Salmon sperm DNA	4.5 µl
P3000™	4.00 µl
Opti-MEM	88.06 µl
Total amount	100.00 µl

Table -2 pcDNA3.1/Hygro(+) plasmid as control

Component	Amount
pcDNAhygro+- plasmid	2.87 µl
Salmon sperm DNA	12.40 µl
P3000™	4.00 µl
Opti-MEM	80.73 µl
Total amount	100.00 µl

Table- 3 two tubes prepared lipofectamine 3000

Component	Amount
Lipo3000	3µl
Opti-MEM	97µl
Total amount	100.00 µl

Gene chip: The HepG2 transfected with, HBSP, were cultured in DMEM with10% FBS in 10 cm plates till a reach 90%confulence .Then the 60 µM of β-sitosterol were added and incubated at 37 °C, 5% CO_2 for 24 hours then the DMEM removed and 1.5 ml of Trizol added to each plate We used Affymetrix gene chip and Agilent gene chip. Total RNA quality control, mark, chip hybridization, washing, staining and scanning, and data extraction and analysis procedure as company protocol. Data extraction and analysis software using the AGCC (Affymetrix® Gene Chip® Command Console® Software) chip fluorescent.

RESULT

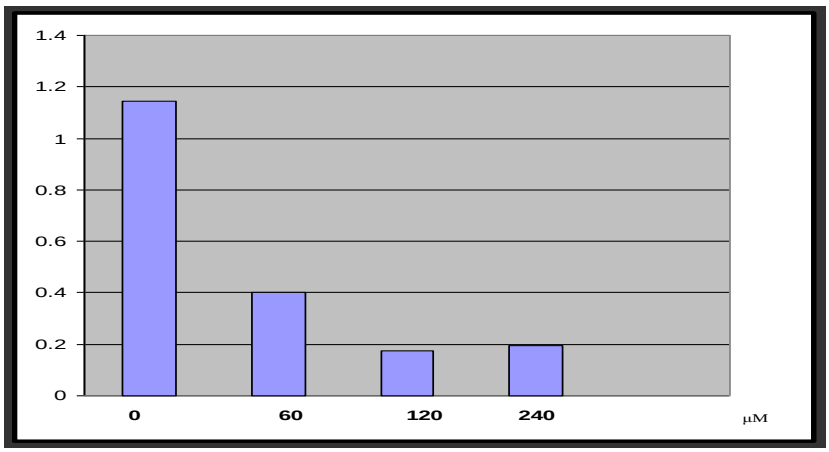

Figure: -1 CCK8 cell viability assay shows the effect of β-sitosterol on HepG2.2.1.5 and the correlation between the numbers of viable cells with the concentration of β-sitosterol, Y- axis shows the number of viable cells. X- axis shows the concentration of β-Sitosterol.

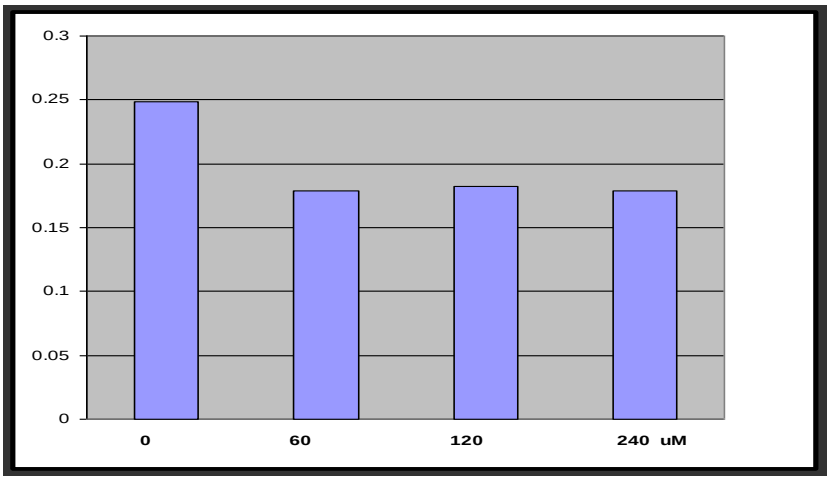

Figure: -2 CCK8 cell viability assay shows the effect of β-sitosterol on HepG2. And the correlation between the numbers of viable cells with the concentration of β-sitosterol ,y-axis shows the number of viable cells x- axis shows the concentration of β-sitosterol

Cell viability: Cell viability by CCK8 result shows decreases in viable cells related to concentration of β-Sitosterol. This was documented by a decrease in the cell viability curve as shown in figure 1 and figure 2.

Cell migration: The numbers of HepG2.2.15 migrant cells treated with β-Sitosterol were found to be less than the control HepG2.2.15 result of cell migration shows the significant decrease in the cells treated with beta sitosterol than control HepG2.2.15 as in figure2

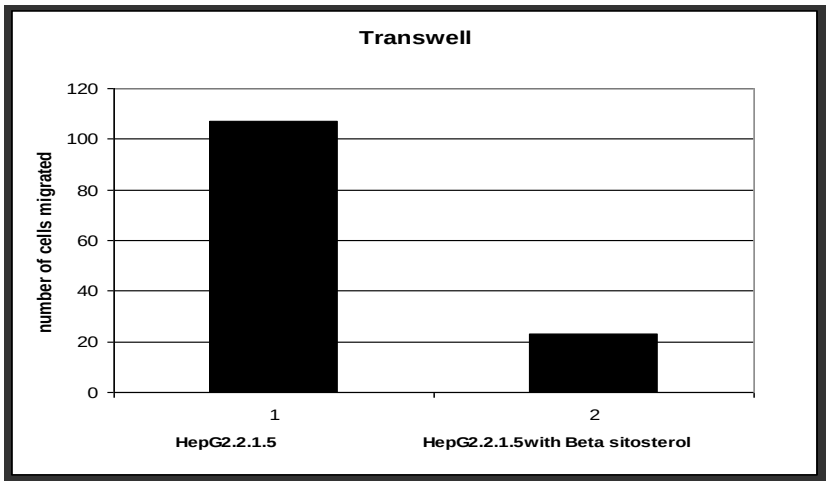

Figure 3 Transwell. β-sitosterol decreased the HepG2.2.15 cells migration. Colum 1 shows the numbers of migrate HepG2.2.1.5 cells as reference Colum 2 shows decrease of the number of migrated HepG2.2.1.5 cells when 60 µM of β-sitosterol added.

Gene expression: Electrophoresis RNA quantification meet the requirement for microarray DNA chip test as shown in figure 4

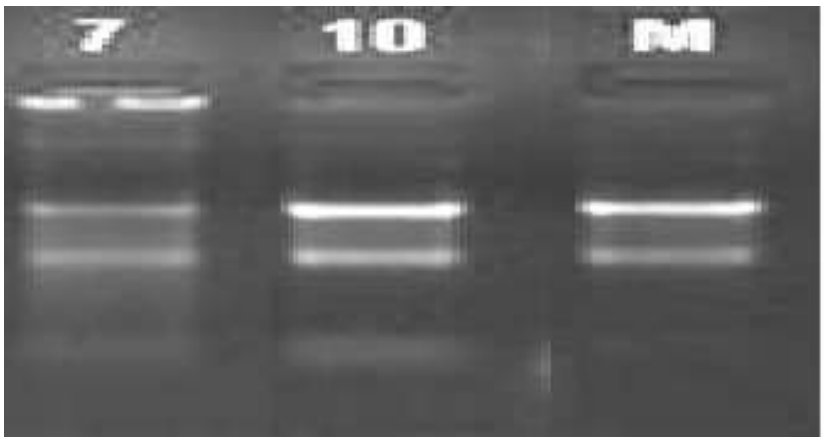

Figure 4 Electrophoresis for quantification of the protein in the samples for microarray test Lane7 HepG2- HBx, Lane10 HepG2- plasmid treated with β-sitosterol, M Lane Hela Cell Control RNA

Table 4 RNA Quantification

Sample ID	Sample name	Sample state	A260/280	260/230	(μg/μL)	mount μg	Sample mass Description
7	HepG2- HBx	TRIzol	1.66	0.79	0.088	1.7	RNA OK
10	HepG2- plasmid	TRIzol	1.87	2.11	0.71	1.6	RNA OK

Gene chip shows significant enriched Gene Ontology (GO) terms in top biological process in HepG2 –HBVx as shown in figure 5

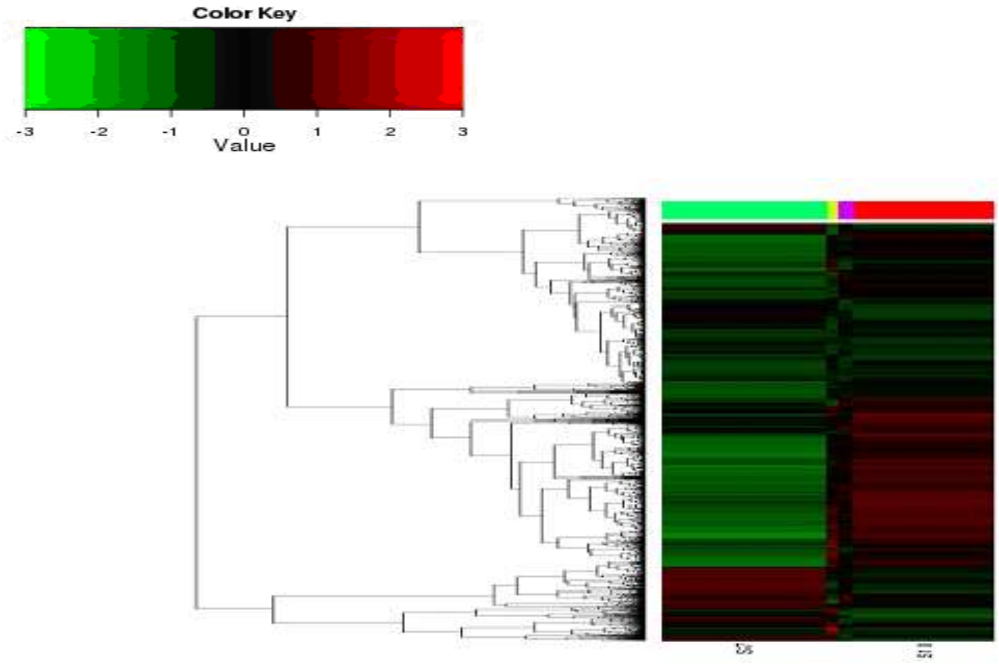

Figure 5 heat map represented genes expression .S7 represented HepG2-HBx , S 10 is HepG2 control both were treated with β- Sitosterol

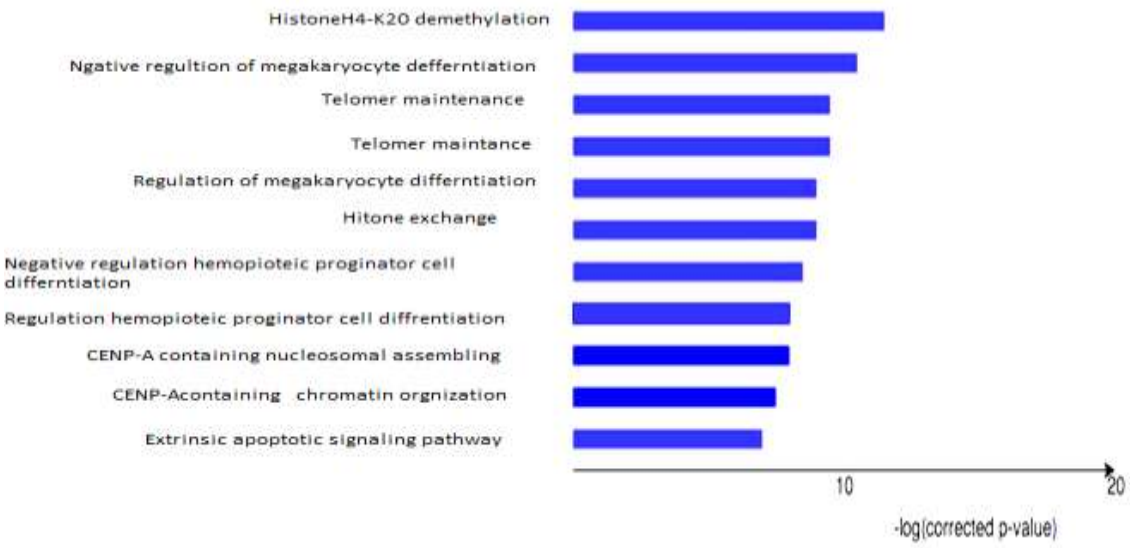

Figure- 6 shows significant enriched biological process log(corrected P value)

DISCUSSION

β-Sitosterol Has effect on hepg2.2.15 by reducing the cell proliferation as well as in cell migration. This result can be a new area of research on hepatocellular carcinoma and hepatitis B virus replication as well as cancer metastasis. We study in deep using gene chip to evaluate the effect of beta sitosterol on HepG2-HBx. The gene expression and Gene Ontology proof there are significant enrichment in negative regulation of megakarocyte, the mature megakaryocte produce thrompocytes[21], and there is a strong correlation between thrombocytosis and clinical tumor biology, found in various types of cancers including HCC.[22-23] β-Sitosterol has negative regulation on megakarocyte which can reduce also the thrombocytosis. The decreases of cell migration have influence in metastasis. has there is a correlation between on hepatocellular carcinoma metastasis and platelet counts .Morimoto and colleagues demonstrated this correlation between extra hepatic metastasis of HCC and high platelet counts.[24] The cancer cells stimulate platelet aggregation and formation, Platelet activation by tumor cells involves molecules as Tissue Factor (TF), adenosine diphosphate (ADP), Thrombin, Thromgouboxane A2 (TXA2) and MMPs, are secreted directly or indirectly stimulated by tumor cells and this mechanism is known as tumor cell induced platelet aggregation (TCIPA).[25] Anti-platelet and Anticoagulant agents commonly used as tumor growth and metastasis inhibitor for many cancer types including liver cancer.[26] We suggested that β-Sitosterol can do that. Telomeres function primarily to mask double-strand break DNA damage signals at chromosomal termini, inhibit terminal exonucleolytic degradation, and prevent chromosomal fusions.[27-28] In somatic cells, and during the cell divisions telomeres shorten with each cell division, and this telomere shortening leads to p53-dependent senescence or apoptosis.[29] association between Histone H4K20demethylation and Histone 4 lysine 20 trimethylation has been found with several cancers, and loss of H4K20 trimethylation has been proposed to be a hallmark of cancer.[30] The changes in biological process of HepG2-HBx due to the effect of β-Sitosterol can be helpful to focus on it as anti-metastasis and impairing HBV replication through its effect on HBx.

CONCLUSION

We conclude that β-Sitosterol has significant effect on hepatocellular carcinoma as well as hepatitis B virus and could be an anti-platelet agents and reduce the HCC metastasis. Also has effect on telomere maintenance and h4k20 demethylation.

RECOMMENDATION

We recommended deep study on histone h4k20 demethylation and telomere maintenance.

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