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Title of Thesis: Transactivity of Hepatitis B Virus X-Protein Influencing the Regulation of Expression of Proteins Involved in Signaling Pathways

Introduction: Human hepatitis B is caused by a small 42 nm virus called Hepatitis B virus (HBV). It involves inflammation of liver tissue, which may progress to liver cirrhosis and hepatocellular carcinoma (HCC). HBx is a gene of HBV that codes for HBx protein. This protein is reported to be responsible for HBV associated HCC development. Mutations are very common in HBV genome, some of which are present in HBx gene and specifically associated with cirrhosis and HCC patients. The role of these HBx mutations in cirrhosis and HCC development is least studied.

Objectives:

- To investigate the nucleotide(s) and amino acid(s) variations within X gene and x-protein (HBx) respectively, among patients with advance liver disease.
- To select suitable cell lines of hepatic and non-hepatic origin.
- To generate various recombinant constructs of X gene.
- To identify appropriate target proteins of signaling pathway(s).
- To investigate the influence of HBx on expression of proteins involved in signaling pathway(s).

Methodology:

HBx was PCR amplified from HBV genome. The amplified HBx was cloned in TA easy cloning vector pTZ57R/T. HBx was then sub-cloned in a mammalian expression vector pcDNA3. Six natural HBx mutations were created in the cloned HBx gene using site directed mutagenesis. All the wild type (wt) and mutant (mt) HBx were then sub-cloned in a GFP tag mammalian expression vector pEGFP-C3. Huh7 cells (human hepatoma cell line) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin and 5% CO_2 at 37^0C. The cells were transfected with the wt/mtHBx constructs using Lipofectamine 2000. Total RNA was isolated from the cells 48hrs post-transfection, using Trizol method and cDNA was synthesized. RT (reverse transcription) PCR and Western blotting were done to check HBx expression in the transfected cells. The effect of wt/mtHBx on the cells was checked by MTT assay and flow cytometry. Gene expression of the host cell, in presence of wt/mtHBx, was studied by Real time RT-PCR (Syber Green). For mitochondrial depolarization and apoptotic studies, cells were stained with TMRE (Tetramethylrhodamine ethyl ester). Readings were taken in fluorescent microplate reader with excitation at 549nm and emission at 575nm. Each

experiment was repeated at least three times. For experiments where graphs were plotted, average values and standard deviations were calculated and graphs were drawn from the readings of three independent experiments on Microsoft Excel data sheet.

Significant Results:

The overall findings of this study lead to the conclusion that most of the HBx mutations studied resulted in high proliferation of target cells when compared to wild type HBx.

A c-terminal truncation mutant of HBx and some point mutations (at nt 1464 and nt 1764 of HBV genome) resulted in increased distribution of cells in proliferating phases of cell cycle (S and G2/M phases) due to fast transition through G1-S check point.

Through the gene expression studies, it was found that HBx is under autoregulation.

A c-terminal truncation and a double mutation (nt 1762+1764 of HBV) of HBx resulted in high cell proliferation and possibly HCC development via transactivating the overexpression of E2F1.

Various HBx point mutants (at nt 1464 and nt 1764 of HBV genome) and a double substitution mutant (nt 1762+1764 of HBV) induced cell proliferation, and possibly HCC growth and progression, migration and extracellular matrix invasion, and metastasis through overexpression of transcription factor c-myb.

A point mutant (at nt 1762 in HBV genome) of HBx resulted in loss of transactivation function of HBx for cyclinA1. This effect was also observed to some extent for some other important host proteins.

Another point mutant of HBx (at nt 1764 of HBV genome) was found to be a second site suppressor mutant that nullifies the effect of mutation at nt 1762 of HBV genome, i.e. restoration of transactivity of HBx for cyclin A1.

All the wt/mtHBx versions resulted in transformation of the transfected cells. The transformation efficiency was highest for the c-terminal truncation mutant as compared to other versions of wt/mtHBx.

The HBx double substitution mutant at nt 1762+1764 of HBV and a point mutant at nt 1753 of HBV genome induced mitochondrial depolarization and apoptosis. This may be involved in development of HBV related hepatic fibrosis and cirrhosis.

In summary, it is concluded that HBx transactivity is very much altered due to the studied natural HBx mutants. The natural mutant of HBx that were specifically found in advanced stages of liver diseases are actually favoring that disease state by transactivating some key cell cycle regulators such cyclin A1, E2F1 and myb, resulting in higher proliferation of the transfected cells. Some of the natural HBx mutations resulted in increased apoptosis of target cells that may be a possible mechanism for the development of fibrosis and cirrhosis in HBV infected patients.