INVESTIGATING THE ROLE OF HBX IN THE DEVELOPMENT OF HEPATOCELLULAR CARCINOMA

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بسم الله الرحمن الرحيم

I dedicate this piece of work
to my loving parents & myself

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LIST OF ABBREVIATIONS

AFP	Alpha-fetoprotein
AP-1	Activator Protein 1
Bcl-1	B-cell Lymphoma 1 protein
$CaCl_2$	Calcium Chloride
cccDNA	Covalently Closed Circular DNA
cDNA	Complimentary DNA
CO_2	Carbon Dioxide
CXCR4	C-X-C chemokine receptor type 4
DMEM	Dulbecco's Modified Eagle Media
DNMT's	DNA Methyltransferases
EDTA	Ethylene Diamine Tetra Acetic Acid
EMT	Epithelial mesenchymal transition
FBS	Fetal Bovine Serum
HBV	Hepatitis B Virus
HBx	Hepatitis B Virus x protein/ gene
HBsAg	Hepatitis B surface Antigen
HCC	Hepatocellular Carcinoma
HIF-1α	Hypoxia-inducible factor 1alpha
HULC	Highly upregulated in liver cells
L.B	Luria Bertani
MAPK	Mitogen-Activated Protein Kinase
$MgCl_2$	Magnesium Chloride
miRNA	microRNA

MTA1	Metastasis Associated protein 1
NCBI	National Centre for Biotechnology
NFκB	Nuclear Factor Kappa Beta
OD	Optical Density
Pen-Strep	Penicillin-Streptomycin
PKB / AKT	Protein Kinase B
TAE	Tris Acetate EDTA
TNFα	Tumor Necrosis Inducing factor alpha
Tm	Melting Temperature
T_A	Annealing Temperature
WHO	World Health Organization
µL	Microlitre
µg	Microgram
Ng	Nanogram
%	Percentage
°C	Degree Celsius
~	Approximately

ABSTRACT

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver and occurs predominantly in patients with underlying chronic liver disease and cirrhosis. The carcinogenesis related to HCC is multifactorial and multi-step process and chronic infection with HBV is considered as one of the etiological agent for the hepatocellular carcinoma (besides many others). Currently many researches have supported the role of Hepatitis B virus X gene and protein (HBx) in the development of HCC. HBx induced carcinogenesis is a result of modulation of various signalling pathways and factors, such as activation of various transcription factors e.g. NF-κB or interaction with cellular oncogenes. This study aims at amplification of HBx sequences from patient samples, cloning poten tionally important X sequence for downstream processes and mammalian expression and transfection of cloned X sequence into HCC cell line. So, the study was designed to screen the HBx protein from blood sera samples of the patients, HBx being cloned in the Ptz57R vector, isolation of plasmid, and the transfection studies were carried out for mammalian expression and also to investigate the role of HBx in the development of HCC through transfection studies. The HCC cells were transfected by the HBx gene cloned in the plasmid vector (pTZ57R/T) and the results indicated the presence of mRNA of HBx in the transfected Huh7 cells as compared to controlled cells. The experiment was successful and HBx gene (cloned in vector) was successfully transfected in HCC cell line. Conclusively, the experiment was successful in amplifying HBx gene from selected patients and the HBx gene (cloned in plasmid) was successfully transfected in HCC cell line. The study will help in studying the role of HBx protein in HCC cell line and development of HCC by transfecting normal liver cells with HBx, better understanding of HBx (HBV etiology) in hepatocellular carcinoma will help in identification and development of potential biomarkers for early diagnosis and treatment of HCC, this will not only help in designing new strategies for the treatment of HCC but also in identification of novel proteins for better understanding of HBx induced HCC.

CHAPTER 1: INTRODUCTION

Hepatitis B virus (HBV) belongs to family of the Hepadnaviridae which usually includes viruses with envelops and they mainly targets the liver and hense can result in acute or chronic infection.The genome of the virus is partially double stranded. (Knipe *et al.,* 2007).

1.1 Etiology and Epidemiology of HBV

In the world the infection caused by HBV is considered to the most common infection caused by any virus (Lavanchy, 2004). Prevalence rate of HBV infection varies according to geographical location and it is different in different regions of the world. In developing countries HBV infection is considered to be endemic, where population size is large and where 95% of the population show HBV infection. Areas where endemicity ratio is high, HBV infections occur mainly in childhood, as majority of childhood infections are asymptomatic so the rate of chronic liver disease and liver cancer are comparatively high (Alter, 2003). Regions like Japan, South America, Middle East, Eastern and South Europe HBV infections are usually intermediately endemic with incidence rate of 10-60%. In developed regions of world like Australia, Northern and Western Europe and North America, the incidence rate of HBV infections is 5-7% with low endemicity (Alter, 2003).

Hepatitis B virus is endemic in Pakistan with approximately 9 million infected people (Ali *et al*., 2011). It is prevalent in our population and the estimation shows it to be 35% to 38% of our population out of which carriers of HBV accounts for 4% (Hakim *et al.,* 2008). The HBV prevalence in healthy adults is 1.4% - 11.0% while the prevalence rate is 2.4% in pediatric population (Ali *et al.*, 2009).

1.2 HBV as an Etiological agent for HCC

Chronic infection with HBV results in development of hepatocellular carcinoma (HCC) along with other etiological agents such as HCV, consuming alcohol, fatty liver , ingesting Aflatoxin B1 and the other factors related with chronic inflammatory and hepatic degenerative changes. Moreover, HBV infection has been reported to cause about one-third of total liver cirrhosis and greater than three-fourth of total HCC cases around the globe (Han *et al.,* 2011). It is transmitted through blood and infected body fluids. As

there exist 6 types of hepatitis virus genotypes that lead to liver inflammation; among which type A, B and C are the most common (Han *et al.*, 2011).

Hepatitis B virus is being considered the major causative agent for diseases in developing countries (such as Pakistan) such as hepatocellular carcinoma, cirrhosis etc. The geographical distribution of viral cases of hepatitis on the basis of type occurring in Pakistan is depicted in figure 1.1.

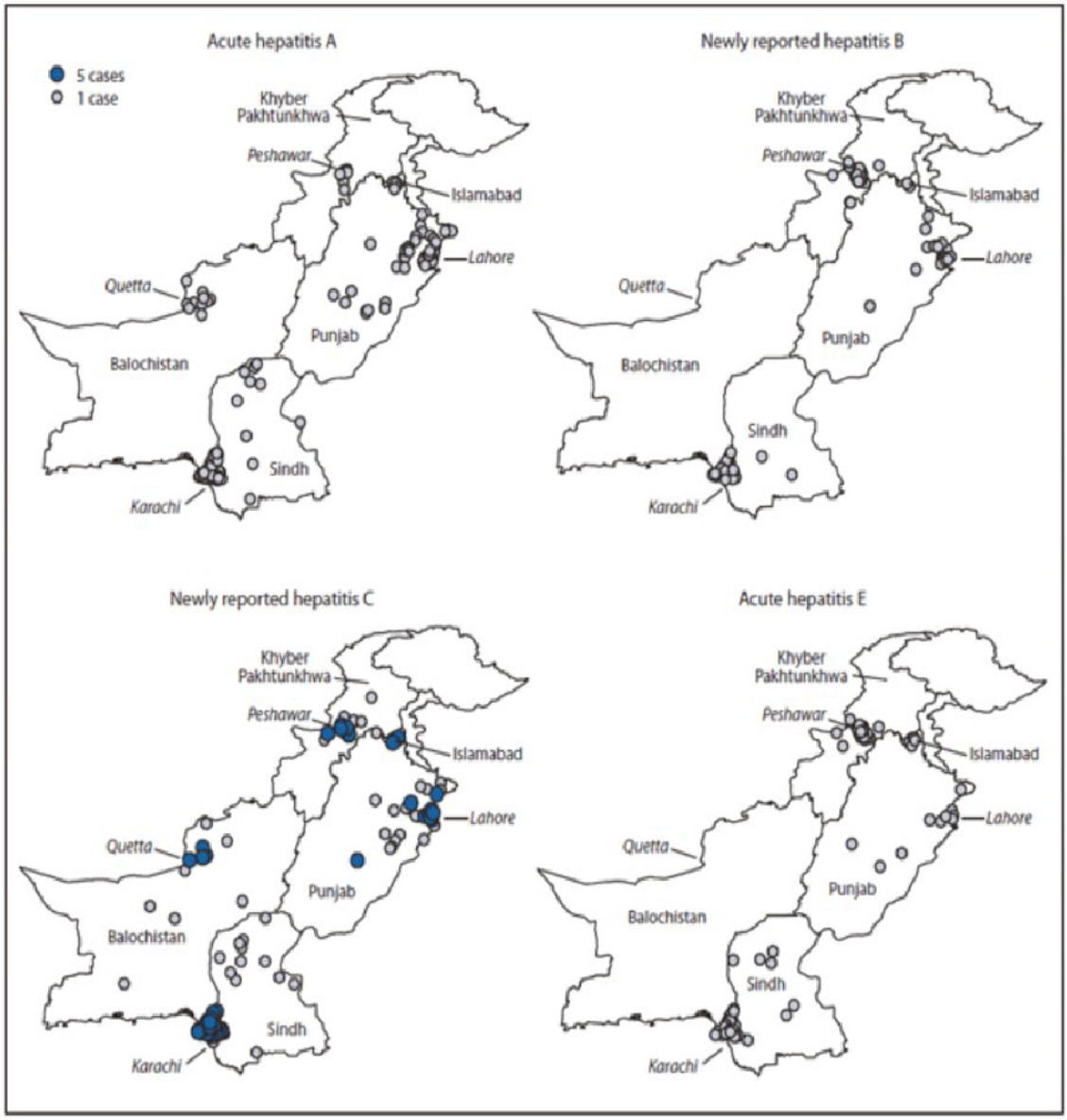

Figure 1.1: Hepatitis cases according to geography (by virus type) in Pakistan (in major cities) during June 2010-March 2011 (Rana *et al.*, 2011)

Hepatocellular carcinoma is considered to be the most common type of cancer and there exists remarkable interrelation in terms of prevalence of HBV infection (chronic,carrier state) & HCC incidence. Globally HBV is considered the etiological agent for 75%-90% of these primary liver cancers (Parkin, 2006).

There are large number of factors which are considered to be risk element for the carcinogenesis of liver and they include, low platelet count (which shows progression towards cirrhosis), high level of alanine aminotransferase (in serum), viral load which is considered to be a critical parameter predicting how HCC develops over the course of time and persistently high level of alpha-fetoprotein which is indicative of elevated cell division rate and mutation and also it shows regeneration conditions of liver (Chen *et al*., 2006; Sherman, 2009) and most importantly, the protein which may play a very critical role in developing the carcinogenesis known as Hepatitis B virus X protein (HBx) (Ganem & Prince, 2004;Yoo *et al.,* 2007).

The hepatic carcinogenesis is the prevailing cause of deaths around the globe and it is considered to be the third potentional cause of death among all the deaths linked to cancer, with estimation of nearly 600,000 deaths per year globally (Chiba *et al*., 2015). On the basis of gender, hepatic cancer be a fifth commonly diagnosed in men whereas in women liver cancer is sixth prime root of cancer-related death around the globe (Wang *et al*., 2013).

The incidence related to HCC worldwide is measured to be around between 250,000 and 1.2 million per year (Chen *et al*., 2006). Individuals having untreated chronic hepatitis B infection (~ 8-20%) develops cirrhosis in time span of about 5 years; out of these adults, approximately 2-5% individuals are being affected by HCC annually and around 20% of these individuals are predisposed to hepatic decompensation. Furthermore, approximately 45% of the cases of HCC and 30% cases of liver cirrhosis are considered to be linked with HBV worldwide. (Hoofnagle, Doo, Liang *et al.,* 2007; Look *et al*., 1991). Therefore HCC is considered to be the most common type of cancer and there exists variations in term of different regions worldwide, in Asians countries (importantly sub-Saharan Africa and China) it is considered to be one of the main malicy, but in majority of the western world it is considered to be less common as shown in figure 1.2.

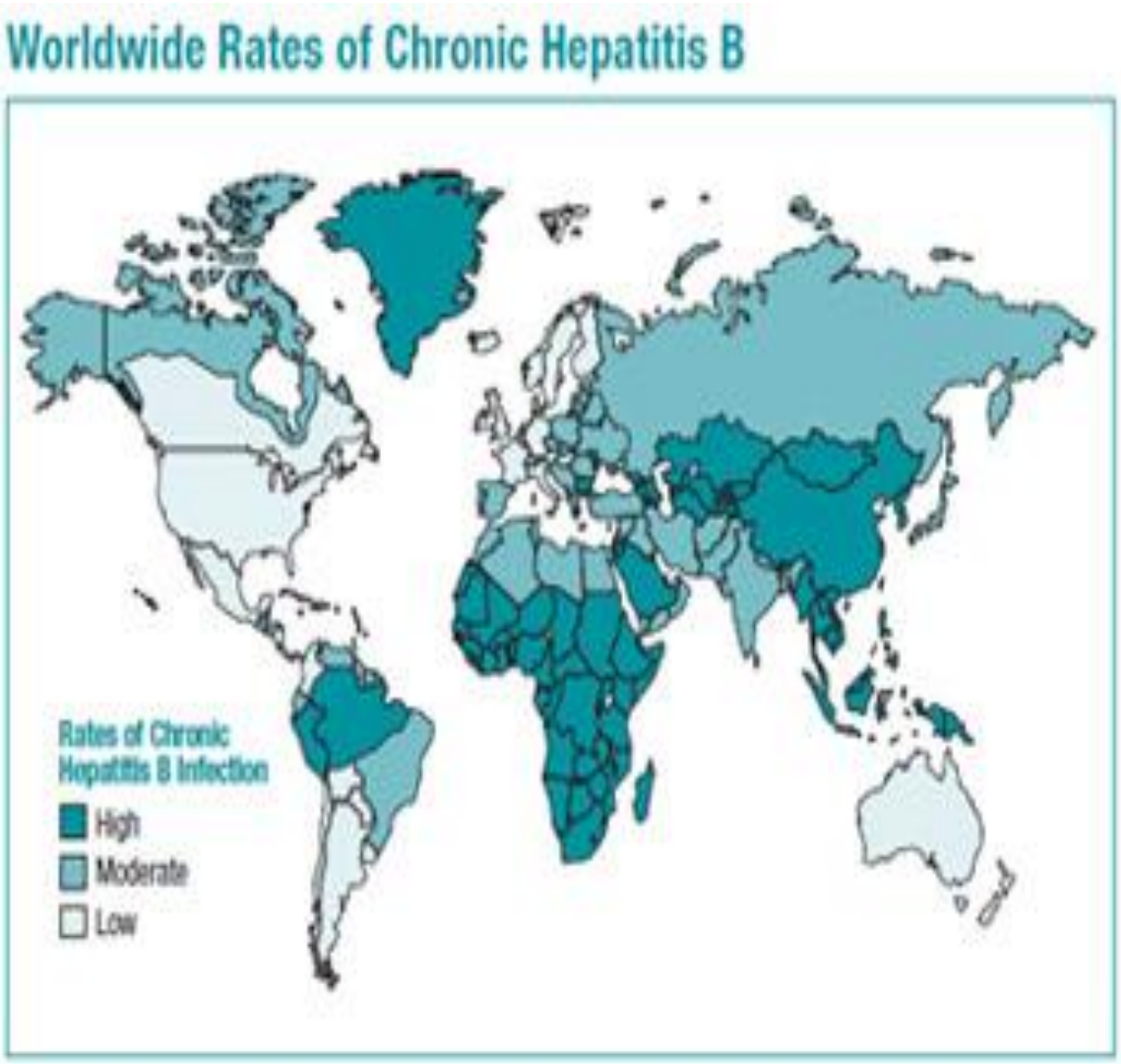

Figure 1.2: Patterns showing levels of chronic Hepatitis B virus infection, as High, Moderate and Low (Retrieved from Centres of Disease Control and Prevention. 2008).

1.3 Diversity of HBV Genotypes

Genotypes of HBV are usually defined by intergroup >8% change in the whole genome and about >4% change in S gene (Okamoto *et al.,* 1988). Up till now total 8 genotypes have been identified. Genotypes A-D were identified earlier and later genotypes E-H were identified (Baptista *et al., 1999*).

1.4 HBV Genome and the Replication cycle

Genome of HBV is hepadnaviral, compact and complex around 3.2 Kb, with its open reading frame being complex and overlapped and is the possible cause of mutations (Norder *et al.*, 2004).

As the genome of HBV is packaged and packaging is in the form of relaxed circular (RC) DNA. It has (+) strand which is bound to proteins i.e DNA-binding proteins and they inhibit its synthesis completely. But these DNA-binding proteins dissociates inside nucleus and ultimately the (+) strand is being extended by viral polymerase and thereby the genome is left as cccDNA. Since DNA strands store energy in their supercoils so virus actually takes benefit from this cccDNA genome, so ultimately it is not dependent on ATP-dependent helicase activity of the host for performing DNA replication.

The genome of HBV contains four genes which has four ORFs which are partially-overlapping includes: surface antigen (S), core (C), polymerase.(P) and X as shown in figure 1.3. Hepatitis B surface antigen (HB s Ag) is coded by S ORF.

There are precore and core regions present in the genes of core (C). For S and C genes, there are present many translation initiation codons (inframe) and they accord the translation process for proteins which are related but they function distinctly (Liang, 2009). Hepatitis B e antigen (HBeAg) is encoded by C at pre-core ORF and at the core ORF it is encoding viral nucleocapsid (HBcAg) (Liang, 2009) .

The large protein i.e the Polymerase (pol) contains total of three subunits: a reverse transcriptase, a subunit having terminal protein and this subunit provides active site for the synthesis of DNA, and the subunit of ribonuclease H and this subunit aids in replication and also delineate RNA which is pregenomic (Liang, 2009). There are two elements in genome, which are controlling the strand-specific synthesis taking place during the process of replication, they are direct repeats 1 and 2 (DR1 and DR2) and are present in the plus strand at 5’ ends. There are another set of elements called as enhancer elements controlling the Liver-specific expression of the gene products which are viral and designated as En1 and En2 (Liang, 2009).

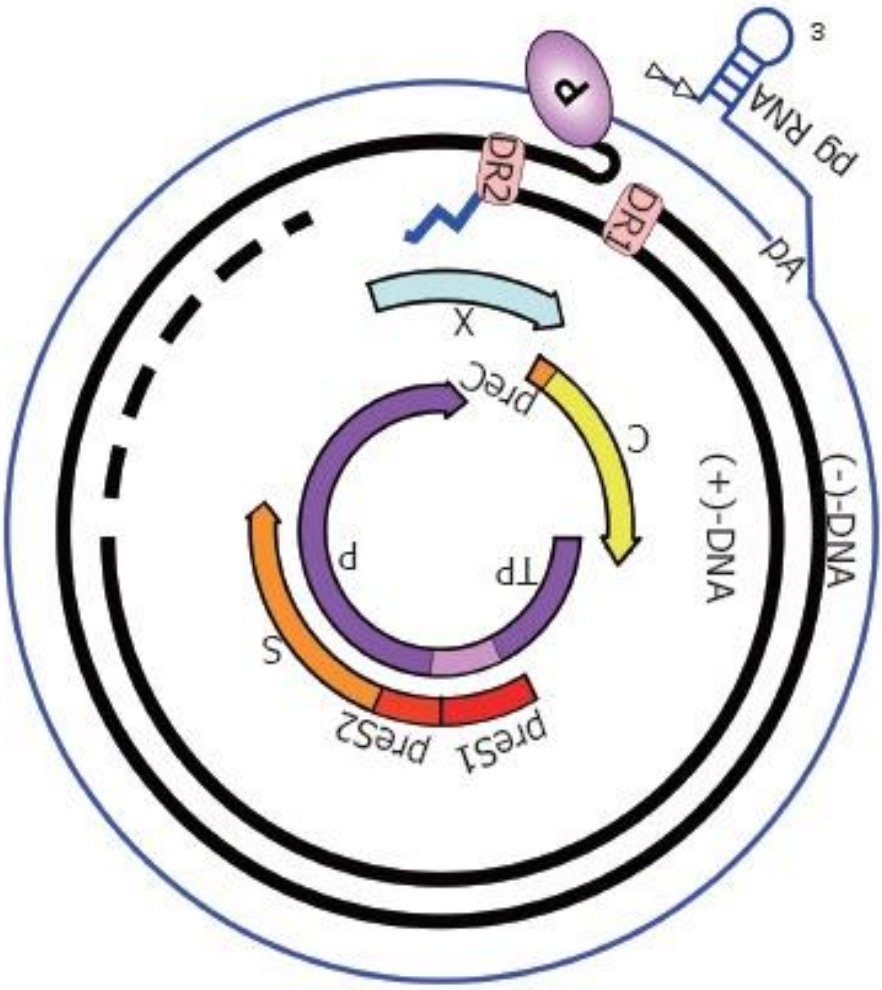

Figure 1.3: Hepatitis B Virus Genome (HBV) organization. The black thick lines are indicative of circular RC-DNA, P shows covalent linkage to the 5' end of the (-)-DNA, and the zigzag line are indicative of linking of RNA primer to (+)-DNA at 5'. Those broken lines parts are indicative of the mixed lengths of the (+)-strands (Beck & Nassal, 2007).

Hepatitis B virus binds to receptors which have not been identified yet and this way enters the cell and is endocytosed. The viral genome makes entry in the nucleus after the coating of the nucleocapsid core has been removed. In the nucleus, the viral polymerase repairs the gap region of single strand, therefore resulting in cccDNA which is the functional template for most RNA polymerase II to perform the process of transcription. Due to the process of alternative splicing in mRNA of HBV, this poses a hurdle in making of small inhibitors which are to be used as target for specific binding domains of specific viruses (Lee *et al*., 2008) .

After the transcription of DNA the mRNA produced may be stored as a pregenomic RNA or it usually get translated into protein. Translation of polymerase (P) and core and also reverse transcription to occur, this Pre-genomic mRNA (pgRNA) acts as the template as shown in figure in figure 1.4. After the synthesis of proteins of virus, the packaging of pgRNA is done by the core. After this process of encapsidation, reverse transcription of pgRNA is carried out by pol and it takes the shape of relaxed circular (RC) DNA which is partially double stranded. To reach the maturity the immature virion starts to make interactions with proteins which are present along the endoplasmic reticulum, afterwards membrane is lysed and and a signal for its release into the extracellular environment has been given (Grimm *et al*., 2011).

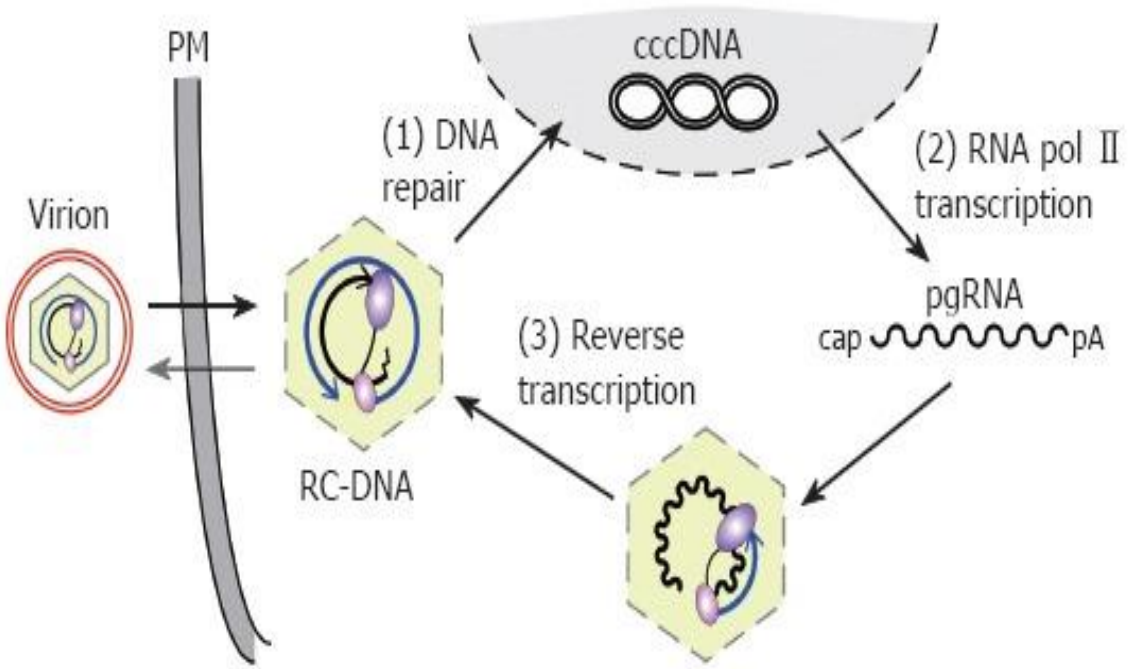

Figure 1.4 Replication cycle of Hepatitis B Virus. Virion infects the cell → RC-DNA releases (cytoplasm) → transported to nucleus →forms cccDNA →(1) Transcription of ccDNA to pgRNA →. (2) pgRNA is encapsidated along with P protein → Reverse transcription → (3) new RC-DNA generated by synthesis of (+)-DNA from (-)-DNA → new RC-DNA generated → new cycle → cccDNA amplification → alternatively RC-DNA (containing nucleocapsid) got enveloped → released as virion (Beck & Nassal, 2007).

1.5 Hepatitis B Virus x protein / gene (HBx) characteristics

Hepatitis B virus x protein (HBx) is a small protein produced by HBV, mainly involves in transactivation of genes and proteins (Yu *et al*., 1999) HBx is considered to be critical for infection produced by virus and as well as for oncogenesis of hepatic cells; though the mechanism by which it causes carcinogenesis still remains obscure. Since then, X protein has been involved in activating various genes (Colgrove, Simon, & Ganem, 1989);(Balsano *et al.*, 1991);(Twu, Lai, Chen, & Robinson, 1993).

As there four genes encoded by HBV and among them one of the critical gene is the X-gene (Koike *et al*., 1988).Amino.acid.sequence.of.154.aa is coded by the HBx and it is mainly conserved in hepadna viruses (mammalian) e.g wood chuck hepatitis virus (W H V) and ground squirrel hepatitis virus (G S H V) (Koike, 1995). It was evident through a research which provide that HBXAg is involved in the HCC came through their work on a transgenic mouse system. (Kim & Koike, 1991; Koike *et al*., 1994). Their results showed the presence of modulated foci, carcinoma with HBx levels being highest that mice system but unfortunately these results can't be reproduced in other mouse systems (Billet *et al.*, 1995). As the role of HBx in HCC remains debatable so HBx has suspected to have oncogenic role.

1.6 Oncogenic role of HBx protein

The X protein (HBx) is widely reported to have involved in malignant transformation of liver cells having chronic HBV infection (Renner, Haniel, Bürgelt, Hofschneider, & Koch, 1995). Most of the time X sequences are found to be truncated at the 3' end.

Hepatitis B virus X protein does not bind directly to DNA, but causes transcriptional activation by its interaction with nuclear transcription factors and modulation of cytoplasmic signal transduction pathways, including the *Ras, Raf*, c-jun, MAPK, $NF_{\kappa}B$, Jak-Stat, FAK, and protein kinase C pathways, as well as *Src*-dependent and phosphatidylinositol-3 kinase signalling cascades (Feitelson & Duan, 1997).

The HBx protein facilitates many critical function that eventually leads to carcinogenesis such as it is involved in inactivating negative growth regulators, favouring the progression of cell cycle and it is also involved in inactivation of tumour suppressor gene p53 expression and other factors too. Although thye mechanism by which HBx is causing HCC is still controversial, so HBx induced carcinogenesis is a result of

modulation of various signaling pathways and factors, such as activation of various transcription factors e.g. NF-κB or interaction with cellular oncogenes like Ras and Srcetc (Kew, 2011).

This study aims at amplification of HBx sequences from patient samples, cloning potentionally important X sequence for downstream processes and mammalian expression and transfection of cloned X sequence into HCC cell line. So, the study was designed to screen the HBx protein from blood sera samples of the patients and to perform transfection studies on HBx by using HCC cell line. The study will help in studying the role of HBx protein in HCC cell line and development of HCC by transfecting normal liver cells with HBx, better understanding of HBx (HBV etiology) in hepatocellular carcinoma will help in identification and development of biomarkers for early diagnosis and treatment of hepatocellular carcinoma, this will not only help in designing new ways to treat hepatocellular carcinoma but also in identification of novel proteins for better understanding of HBx induced HCC.

CHAPTER 2: LITERATURE REVIEW

The HBx protein has been observed to play a vital role in transforming hepatic cells which are predisposed to chronic infection and it was confirmed by the presence of 2/3rd of DNA (HBV) which contains HBx sequences in the hepatic cells (carcinomic). (Renner, Haniel, Bürgelt, Hofschneider, & Koch, 1995) . Most of the times X sequences at 3' end are truncated.

The Hepatitis B virus X gene has an ORF of around 465 base pair and this ORF is being translated into a 154-amino acid (aa) protein. The *HBx* gene is present in the proximity of viral genome i.e at the sticky end and is present upstream of C gene. It overlaps with many other genes which are mainly Pre C, ORF5, ORF6 and viral polymerase. As this gene itself cannot attach directly to the DNA helix, so as a result it can cause activation of several other protein factors and allow them to bind to their own promoters or any other enhancers and promoters. There, HBx is responsible for transactivation function (Seeger & Mason, 2000) as shown in figure 2.1.

Moreover, HBx gene at its the 5' end lap over with ORF of DNA polymerase P (Baptista *et al*., 1999; Venard *et al*., 2000). Therefore, in the viral genome, the X gene has got the stretched out overlapping region present in between the functional and structural sequences. Fundamentally, due to the presence of overlap between the regulatory elements and coding regions the X gene, so any of the mutation in the DNA or any deletion in the DNA mutation can influence both the transcriptional regulation and the gene at functional level.

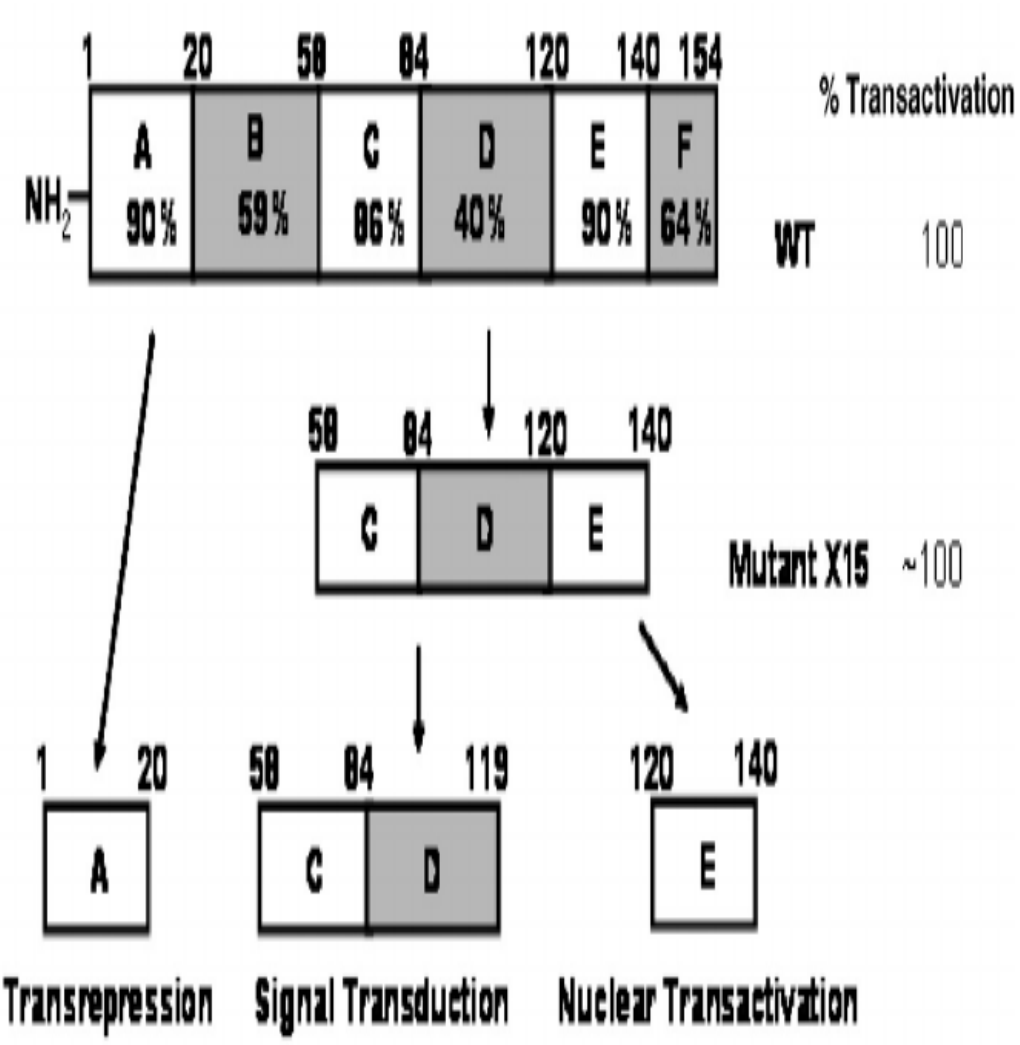

Figure 2.1: Domain structure of Hepatitis B virus x (HBx) gene with some putative functions (Kumar *et al*., 1996).

Currently through many researches it has been obvious that HBX gene/protein have role in causing the carcinogenesis of hepatic cells. Hepatitis B virus X protein is known to be multifunctional as this protein is involved in modulating the replication of HBV, various signal transduction pathways, cell cycle, apoptosis, and transcription as shown in figure 1.7

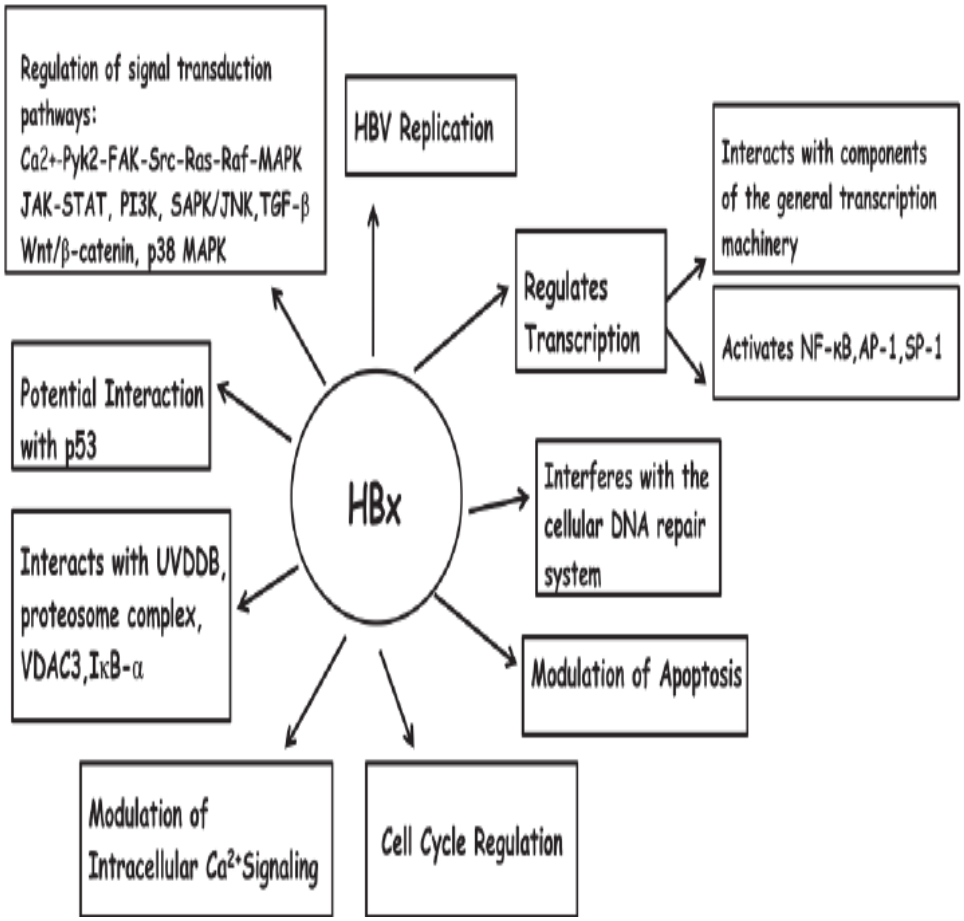

Fig 2.2: Schematic representation of HBx protein activities. It is is known to be multifunctional as this protein is involved in modulating the replication of HBV, various signal transduction pathways, cell cycle, apoptosis, and transcription. Any of these properties are actually attributers towards HCC (Benhenda *et al.*, 2009; Bouchard *et al*., 2001).

Moreover, HBx gene can be used to transfect human HCC cells and it can be transcribed in them because it can easily incorporate in the host genome, although this fuction can be performed by gene without replication being taking place (Hwang *et al*., 2003; Peng *et al*., 2005). As HBx protein has the property that it doesn't binds directly to double helix so instead it activates several enhancers and promoters (Zhang *et al.,* 2014).

In all mammalians hepadnaviruses this HBx gene is greatly conserved and it usually codes for a small protein and this protein is usually be expressing at very low level when the HBV infection is taking place (Yen, 1996).

It has been shown in studies that HBx is actually involved in transactivation of such genes which are incharge of regulation of growth of cell so it suggests that HBx may stimulate uncontrollable cell growth (Yen, 1996).

Considering this idea it has been observed that HBx is involved in stimulating the growth in inactive fibroblast cells (Koike *et al.*, 1994), and it is also involved in de-controlling cell cycle checkpoints (Benn and Schneider, 1995). Moreover, in some transgenic mice the carcinogenesis is induced by HBx (Kim *et al.*, 1991).Furthermore, by the view of certain genetic terms, some of the transgenic mice either shows no pathology towards HCC (Lee *et al.,* 1990), just higher tendency towards carcinogens (chemical) (Slagle *et al.,* 1996), or higher rate for HCC induced by c-myc (Terradillos *et al.,* 1997). Therefore, the real effect of HBx on liver oncogenesis and its mode of action still remain ambiguous. The notion that HBx perform transactivation function in the life cycle of virus and carcinogenesis is still not cleared yet but it is generally believed that HBx is really important for such functions.

2.1 Different studies highlighting signalling pathways affected by HBx:

The process by which HBx brings about malignant alterations, is still obscure, but as a result of recent studies this has clearly been shown that HBx has very vital role in HCC (Hwang *et al.,* 2003).

As this fact has clearly been demonstrated about binding of HBx to DS DNA that it does not bind the DNA (double stranded), so it mainly be playing role as a transcriptional co-activator and puts pleiotropic effect on several cellular functions. HBx stimulate transcription of many genes such as viral genes and c-myc gene in a cell culture system (Henkler & Koshy, 1996; Su *et al.,* 2007).

This protein (HBx) is also found to function in different cell signalling pathways of cytoplasm. As HBx is mainly located in cytoplasm, so here it shows interactions and also activates different protein kinases (IKK, PI-3-K, protein kinase C, protein kinase B etc). HBx has also been shown to stimulate the high-level amplification of centrosome and also induces mitotic mutations (Yun *et al.,* 2004) and is depicted in figure 2.3

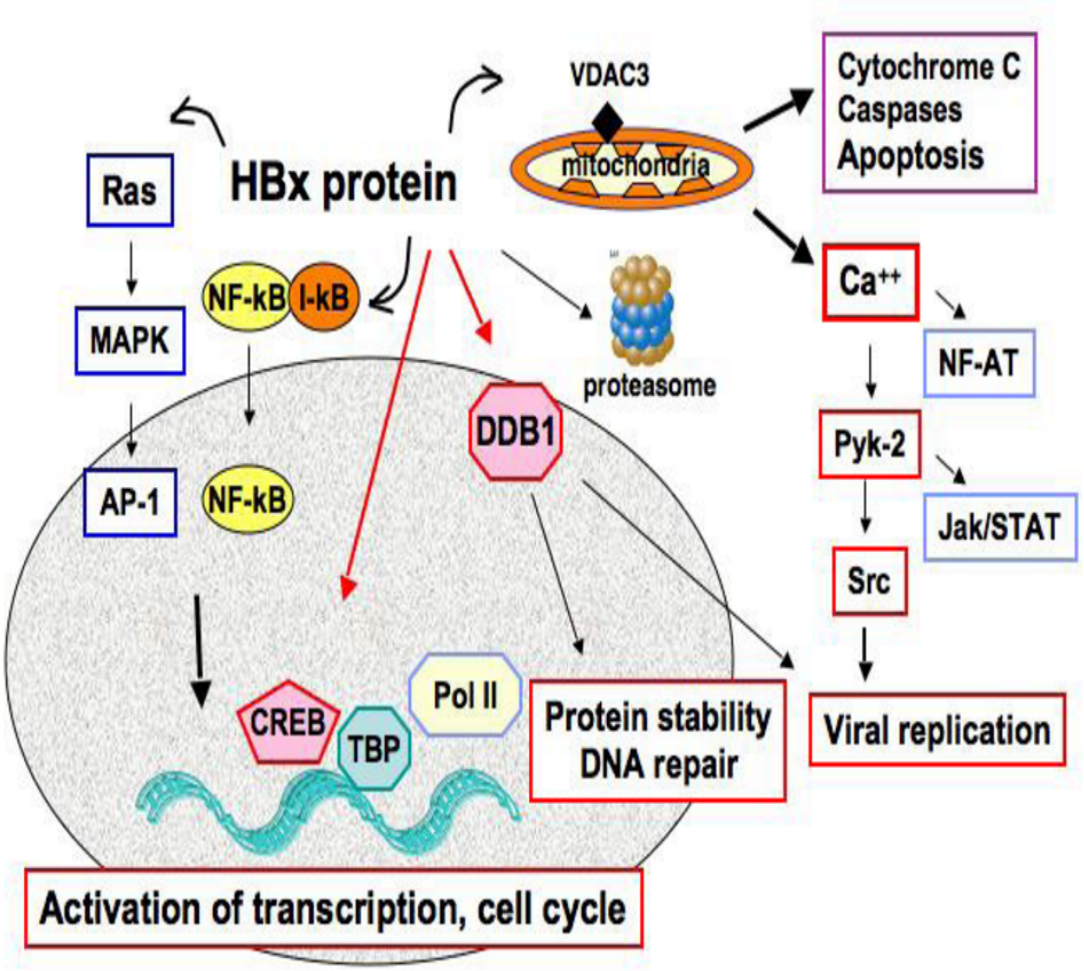

Figure 2.3: Large array of HBx functions that constitute its pleiotropic activities eventually leading to cellular transformations via trigger of virus replication, modulation of cell cycle, stimulation of signalling pathways, perturbing of apoptosis and interference with DNA repair, while all HBx-interactions taking place in both cytoplasm and nucleus (Benhenda *et al.*, 2009)

Nonetheless, HBx has also been involved in stimulation of several elements of transcription which includes Nuclear factor kappa B, apoptotic protein 1, apoptotic protein 2 etc. It has also been shown to affect the several elements of transcription in the cells of host and in this way generate various cellular responses (Zhang *et al.,* 2005).

Below is the review of different signalling pathways and studies which demonstrates the role and mechanism of HBx in the process and transformation of cells, ultimately leading to carcinogenesis.

Signalling pathways are mainly involved in oncogenesis. During the course of HCC, HBx interacts with various signal transduction pathway and plays the role of a crucial carcinogenic protein shown in figure 2.4. In the past majority of studies carried out just highlighted the expression of HBx in comparison to important molecules in several signalling pathways. But with recent advances in knowledge and research, so the mechanism which is causing changes in expression is also illustrated.

Figure 2.4 The most Important and Functional description of HBx while depicting its part in development and metastasis of HCC. These schematic events shows carcinogenic mechanism describing the important oncogenic features of HBx in the causation of carcinogenesis in hepatocellular Carcinoma. HBx stimulates many crucial molecules thus promoting metastasis in hepatocellular carcinoma. Furthermore, some of the independent risk agents such as pre-S deletions, DNA titer, X/precore mutations, HBV genotypes,

HBsAg status and HBeAg status are also responsible for Hepatocellular Carcinoma (Behenda *et al.,* 2009).

2.1.1 HBx and the Notch signalling pathway:

Past studies have shown that the carcinogenesis of liver has been caused by the Notch pathway but there is no proper specification that which specific transmembrane receptor i.e. Notch1 to Notch4 has been involved.

In one study, it has been shown that there is a connection between Notch pathway and HBx and it has been shown by taking in view that how HBx infected cells have been affected by γ-secretase inhibitor. Nonetheless, it was quite difficult to predict that which receptor has been interacting with the HBx as all of the Notch receptors got blocked.

2.1.2 PI3K/mTOR pathway:

In the process of hepatocarcinogenesis, PI3K/mTOR pathway is also shown to be essentially crucial. In the recent research it has been shown that HBx stimulates the upregulation of α-fetoprotein (AFP) which in turn get attached to PTEN (phosphatase and tensin homolog) and in this way debilitate its interdiction on PI3K/mTOR pathway (Zhu *et al.,* 2015).

2.1.3 Tumour Suppressor Gene p53 and HBx:

The most common type of mutations present in almost every kind of cancer are usually be associated to tumour suppressor gene p53.In the carcinogenesis of HCC, p53 plays the crucial role. According to different studies there is an important transactivation link between p53 and HBx and as a result of which p53 is repressed by HBx by binding to C-terminus of p53 (Elmore *et al.,* 1997).

Moreover, in a study it was found out that the downstream signalling of p53 and its expression is constrained by mutant type HBx and and is not done by wld-type HBx (Kew, 2011).

2.1.4 HBx and the Nuclear Factor kappa B Signalling pathway

One of the most important transcriptional factor in oncology is Nuclear factor (NF)-κB and it makes different connections with different signalling pathways and in this way it act as a controlling factor for initiation of tumour, its developing phase, the invasion phase of tumour and its spread through tissues and organs. The metastasis-

associated protein 1 is the most important modulator of chromatin and it is involved mainly in induction of tumour and inflammation. NF- κB interfere with the Notch signal pathway and in in this way inhibiting the Notch 1 cascade in the normal hepatic cell line L02 through the transfection of HBx (Luo *et al.,* 2013).

Moreover, the down regulation of MMP14 (matrix metalloproteinase 14), VEGF (vascular endothelial growth factor) and MMP9 is through the blockade of NF-κB signaling carried out by HBx and this is repressed by PDTC.

2.1.5 HBx and Methylation

Recently a study demonstrated that HBx has the ability to transactivate the DNMTs (DNMT1 and DNMT3A) and in this way it upregulated their level (Tian *et al*., 2013). It was demonstrated that HBx downregulates miR-101eventualling causin methylation of DNA to be deregulated caused through miR-101-targeting of DNMT3A which promotes carcinogenesis (Wei *et al.,* 2013). According to a similar study HBx upregulates both DNMT1 and DNMT3A at transcriptional as well as translational level, subsequently leading to p16 (INK4A) induction promoter methylation ultimately inhibiting expression of p16 (Zhu *et al.*, 2010) as shown in figure 2.5.

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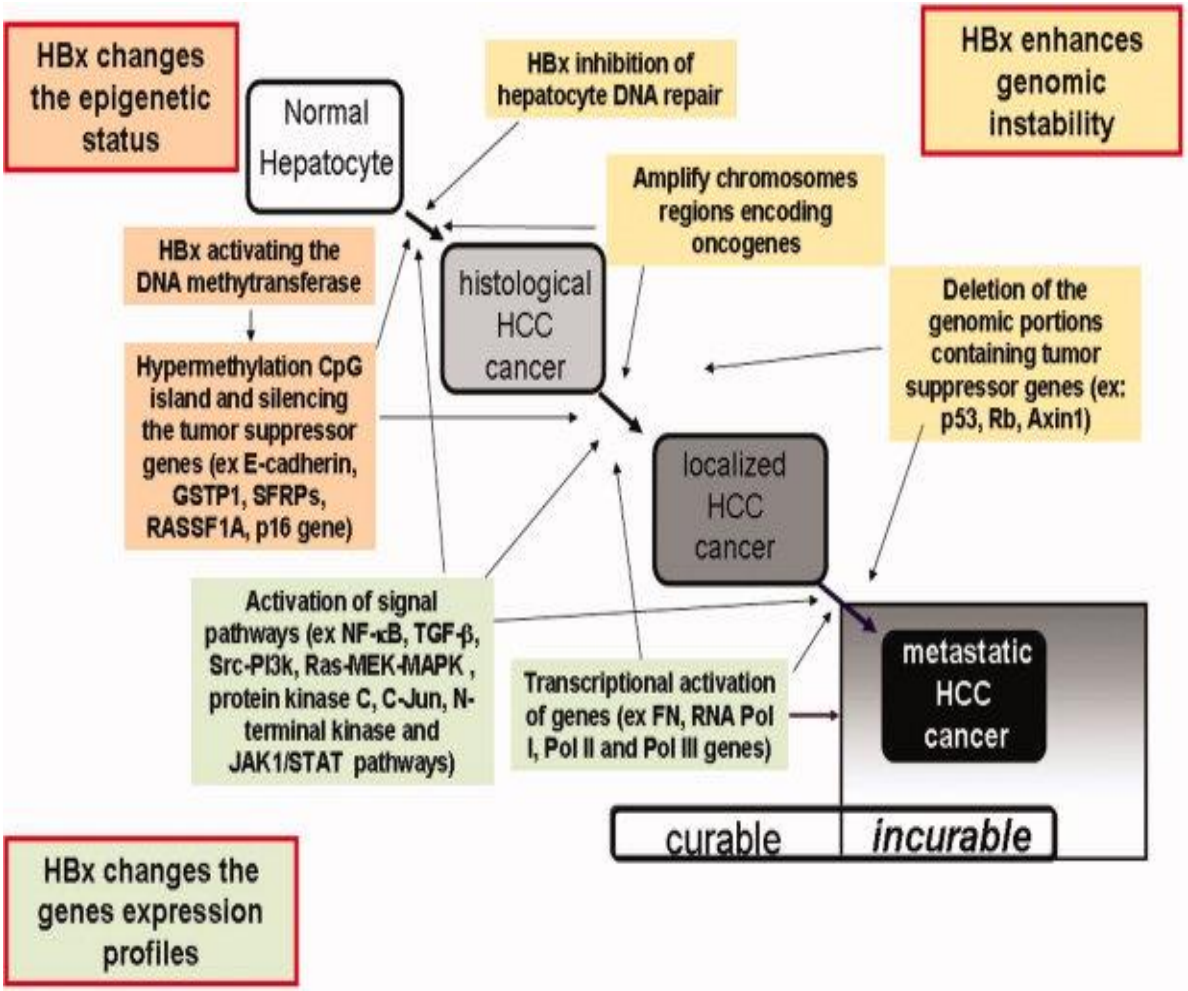

Figure 2.5: Hepatocarcinogenesis induced by HBx and done through alterations in epigenetic status, inhibition of DNA repair in hepatic cells, Genomic instability etc (Zhong *et al.,* 2002).

2.1.6 Apoptosis induced by HBx

As hepatitis B virus x protein is responsible to initiate apoptosis, due to which the carcinogenesis of hepatic cells occur in rather two distinct ways and this largely depends on different factors with which HBx shows interactions. More specifically there are various mechanisms which are responsible for the apoptosis initiated by HBx.

2.1.6.1 Pro-Apoptosis

TRAIL also known as TNF-alpha related apoptosis inducing ligand functions in inducing apoptosis or cell death majorly in cancer cells. HBx stimulates or upregulates one of the death inducing receptor known as TRAIL-R2 (DR5) and in this way HBx is responsible for apoptosis through TRAIL.

Moreover Nuclear Factor Kappa B signalling is also involved in increasing the expression of DR5 (Kong *et al.,* 2015). The function of caspase-8 ubiquitination can be altered by A20 which is actually an E3 ubiquitin ligase. Actually, this caspase 8 is a very crucial intermediant of TRAIL signalling and its alterations can result into changes/ inhibition of apoptosis.

The upregulation of A20 is blocked by HBx by increasing the miR-125a expression which in turn reduces the inhibition of caspase 8 and therefore hepatic cells become sensitive to cell death induced by TRAL (Zhang *et al.,* 2015).

2.1.6.2 Anti-Apoptosis

For the blockade of apoptosis, the two genes Mcl-1(myeloid cell leukemia 1) and Bcl-2 (B cell lymphoma 2) are involved and causes their upregulation but at the same time HBx reduces the expression of Bcl-2-associated X protein (Bax) (analogue of Mcl-1 and Bcl-2), hepatic progenitor cells.

Moreover in a study it was found that there was increase in Beta-catenin and reduction of caspase-3 and caspase-9 which clearly depicts that in progenitor cells, HBx inhibits cell death.

So this shows that HBx performs both functions of apoptosis and an anti-apoptosis (Shen *et al.*, 2013).

CHAPTER 3: MATERIALS AND METHODS

3.1 Sampling

The sampling was carried out and 90 blood sera samples were collected: Out of 90 blood sera samples 50 samples were taken from Diagnostic Lab of Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST, Islamabad and 40 from Shalimar Hospital, Lahore and Itefaq Hospital, Lahore. During sampling blood was collected in EDTA tubes and ice packs were used as storage medium for transportation purpose.To obtain the sera, blood samples were subjected to centrifugation for 8 minutes at 2500xg in 1.5mL centrifuge tubes.The top layer was collected and transferred to fresh tubes.Moreover, the blood sera samples collected from patients were tested to be positive for HBs.

3.2 Viral DNA extraction

For extraction of viral DNA from the sera samples PureLink Viral RNA/DNA kit (Invitrogen,USA) was used.The protocol followed was the manufacturer's protocol, according to which in a fresh microfuge tube 25µL of Proteinase K was added.To this tube 200µL of serum (cell-free sample) and 200µL of Lysis Buffer was added.The tube content was vortex and then incubation was carried out for approximately 15 minutes at 56°C. 250µL 100% ethanol was added to tube and vortexing was done again. The lysate was given the time for incubation at RT for 7 mins. Purification was carried out by transferring the lysate in a collection tube of Viral Spin Column. Centrifugation was at 6800xg for 60 seconds was carried out, the collection tube was discarded and the spin column was transferred to new tube. 500 µL of Wash solution (containing Ethanol) was used to wash the column and centrifuged at 6800 xg for 1 minute. In the step washing was carried out again and flow was discarded. The column was transferred to new Wash tube and centrifuged for 1 minute at maximum speed so that any residual Wash solution could be discarded. The spin column was transferred to a new Recovery tube and added to the column. To a new recovery tube spin column was transferred, 50µL Elution Buffer was transferred to it and incubation was carried out at RT for 60 seconds. The tube was centrifuged at maximum speed for 1 minute so that nucleic acid would be eluted. The spin column was discarded and the purified viral DNA wasused in detection protocols and also kept at -20°C to be used in future.

3.3 Detection of purified viral DNA through Nested PCR

In order to confirm the presence of purified viral DNA, detection PCR was carried out. It was a Nested PCR based on 2 rounds of amplification and 2 pairs of primers and also set of genotyping primers consisting of universal outer primers, universal inner primer (antisense) and sense primers were used to detect the viral DNA(Naito *et al*., 2001). Sequence of primers is given in table 3.1.

Table 3.1: The primers and their sequences used for Detection of HBV DNA

Sr. No.	Primer Code	Sequence 5' → 3'	Length
1	MTB5	CATCCTGGTGCTATGCCTCATCT	23
2	MTB6	GGTATGTTGCCCGTTTGTCTTCT	23
3	MTB7	GGCACTAGTAAACTGAGCCA	20
4	MTB8	CGAACCACTGAACAAATGGCACT	23
5	P1	TCACCATATTCTTGGGAACAAGA	23
6	SI-2	CGAACCACTGAACAAATGGC	20
7	B2R	GGAGGCGGATYTGCTGGCAA	20
8	BD1	GCCAACAAGGTAGGAGCT	18
9	BE1	CACCAGAAATCCAGATTGGGACCA	24
10	BF1	GYTACGGTCCAGGGTTACCA	20

The Nested PCR consisted of two rounds of PCR, in each cycle a 25 µl of mixture for the PCR containing 1X buffer (Taq Polymerase), 2 mM magnesium chloride ($Mg\ Cl_2$), 2 mM dNTPs, forward and reverse primers (10pM each) and thermostable Taq Polymerase (1U)were used.For the first round of PCR the template used was taken from the aforementioned viral DNA extraction protocol, however for second round of PCR making use of another pair of internal primers, the PCR product of first round was used as template. To make up the final volume upto 25 µL nuclease free water was used. Profiles used for thermocycler for round 1 and round 2 were: Denaturation initially for 3 mins at 95°C, second step denaturation for 45 seconds at 94 °C, primer annealing at 64 °C for 45 seconds, extension for 1 minute at 72 °C for 30 cycles and final extension for 5 minutes at 72 °C and in 2^{nd} round initial denaturation for 3 minutes at 95 °C, denaturation

for 45 seconds at 94 °C, primer annealing at 52 °C for 45 seconds, extension for 1 minute at 72 °C for 30 cycles and final extension for 5 minutes at 72 °C.

Using the standard PCR protocol, the DNA samples were subjected to PCR reactions using thermocycler (Swift Maxi- Esco Swift MaxPro thermal cycler) and amplification of~200 bp fragment of HBV genome was obtained.

After the second round of Nested PCR, the product was analyzed using 2% of agarose gel. In 1X Tris Acetate EDTA (TAE) the agarose gel was dissolved for the preparation of gel. In distilled water Tris-base (242g) was added, also glacial acetic acid (57.1 mL) 0.5 sodium EDTA (100 mL) and filled upto the mark with distilled water to makeup 1L. 1X TAE was prepared from the stock solution TAE (50X) by diluting the 20 µL of stock TAE in 980 µL of distilled water. In 80µL of this working solution of TAE, 1.6 g of agarose was added and placed in microwave. When the temperature of the mixture gets low by keeping at room temperature then ethidium bromide [(5 µL)(10mg/mL)] was included for visualizing the gel. For loading purpose ~5 µL of the product was taken along with 1X loading dye and run on the gel at the current 85A for about 30 mins. To determine approximate size 100bp ladder was also loaded. After the running time gel was visualized in UV-transilluminator and then photographed in gel documentation system.

3.4 Amplification of HBx Gene by Polymerase Chain Reaction

The extracted DNA which gave positive results in the detection of HBV were subjected to PCR to amplify the HBx gene of amplicon size of 465bp (approximately).

For the amplification of HBx gene primers were designed by obtaining complete sequence of HBx from GenBank of National Centre for Biotechnology Information (NCBI) (NCBI reference number YP_009173867.1). While designing primers cloning was taken into consideration for the sake of future use. BLAST tool and Clustal W software of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used for homology search for finding the specificity of primer for gene. For the sake of directional cloning restriction sites were added to be used in future. The restriction sites for forward primer were ApoI, EcoRI restriction enzymes. Restriction sites for reverse primer were AsciI, HaeIII, EagI restriction enzymes and are underlined in the primers sequences present in table 3.2. Both primers have shown 100% sequence identity with the HBx gene and GC content, Tm and self-complementarity was checked through online tool OligoCalc. The sequences and properties are shown in (Table 3.2):

Sr. No.	Primer Code	Sequence 5'→3'	Tm (°C)	% GC Content
1	HBXDF2	CTTGAATTCGTTATGGCTGCTAGGC TGTG CTGCCAA	71.5	54.3
2	HBXDR	GTTGCGGCCGCTTAGGCAGAGGTGA AAA AGTTGCA	72.9	50

Table 3.2: Primer sequences for amplification of HBx gene.

3.4.1 Optimization of PCR

The gradient PCR was carried out to optimize the annealing temperature for amplification HBx gene, the gradient PCR was carried by using the annealing temperature from 50°C to 60 °C at 2 °C intervals.The annealing temperature for HBx was optimized at 52°Cand was used for further reactions for the amplification of HBx.

For HBx amplification the reaction mixture was prepared (25 µL) PCR reaction mixture was prepared that consisted of Taq Polymerase buffer (1X), magnesium chloride ($MgCl_2$) (2 mM), dNTPS (0.16mM each), forward and reverse primers (20 pmol each), thermostable Taq polymerase (1U) and template (viral DNA) to make up the volume; PCR water was used. In thermocycler the mixture was placed and following protocols were followed: Denaturation initially for 5 minutes at 95 °C, second step denaturation for 40 seconds at 94 °C, annealing at 52 °C for 40 seconds, extension for 50 seconds at 72 °C for 30 cycles and final extension for 10 minutes at 72 °C.

1.2% agarose gel was prepared to analyse the PCR products.

3.5 Cloning of HBx gene

3.5.1 Ligation in TA vector

Amplified products were ligated into TA vector and following mixture was prepared; 5 µL of purified PCR product (gene of interest), 1 U of T4 DNA ligase, 2 µL of vector and NF water used to make up the total of 10 µL volume. This mixture of ligation was incubated for overnight at 16 °Cand the next day transformation was carried out.Figure 3.1 shows the map of the vector and site of HBx cloning.

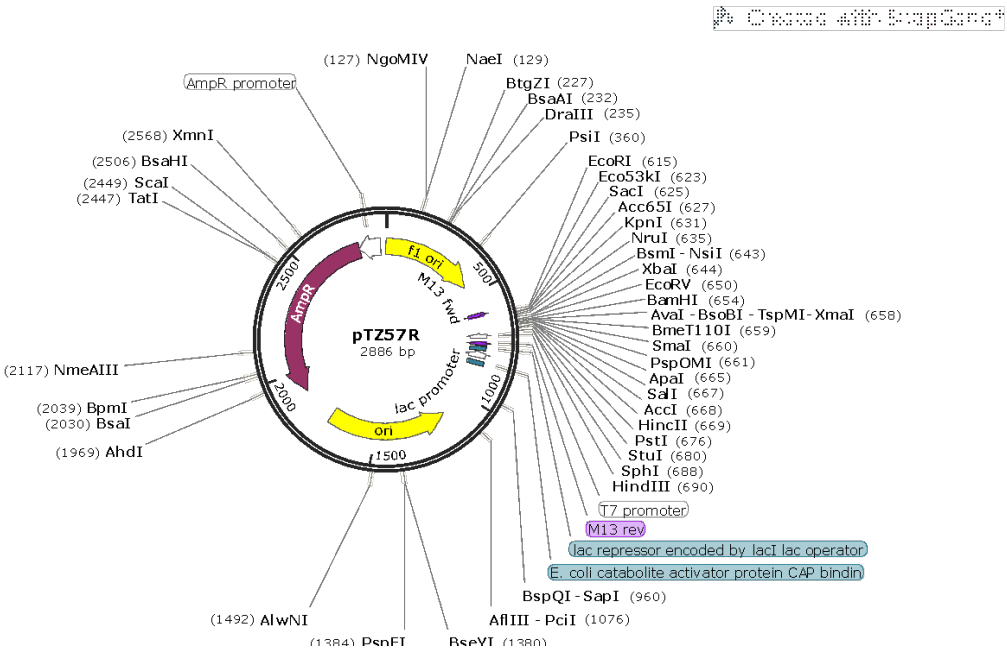

Fig 3.1: Vector Map and HBx cloning site

3.5.2 Transformation

DH5α strain of Escherichia coli (E. coli) was transformed with the ligation mixture.

3.5.3 E. coli competent cells

E. coli competent cells were prepared following the standard protocol (Thermo Fisher US)

3.5.3 Transformation

For the transformation heat shock method was used, as explained by Woodcock *et al.,* (1989). In competent cells (100 µL) ligation mixture (5 µL) was transferred and incubation for 30mins 0n ice was carried out. Afterwards the heat shock was carried out on cells for 2 mins at 42 °C & promptly transferred to ice for 2-3 minutes for cooling. After the addition of LB medium (without Ampicillin) incubation was carried out for duration of 60 mins at 37 °C to allow cells to grow and for the expression of antibiotic resistance gene. After incubation, centrifugation was carried out for 2 minutes at 14,000 rpm for harvesting the cells.On the plates 100Mm IPTG (40 µL), X-Gal (40 µL) were spread out so that LacZ gene could be induced and this would aid in blue/white screening.

The harvested cells were spread on ampicillin containing LB agar plates (200 µg/ mL). For the optimal bacterial growth, incubation was carried out at 37 °C overnight.

3.5.4 Selection of clones

For the purpose of selection and screening of clones, colonies were picked for selection purpose and approximately 4-5 white were taken and inoculated in LB media containing Ampicillin(3 mL) (in a ratio of 200 µg per mL) incubation of 16 hours was carried out at 37 °C to allow the optimal growth of cells. Restriction digestion was done to confirm the clone.

3.6 Plasmid isolation

For plasmid isolation, 100µL of starter culture of positive clones was suspended into 10 mL of L.B media containing Ampicillin and incubated at 37 °C overnight at constant shaking. After incubation, it was centrifuged at maximum speed for 2mins. Centrifugation was repeated twice or thrice for the cultured media and everytime supernatant was wasted out. GeneJet Plasmid Miniprep Kit (# K0502, Fermentas, USA) was used for isolating plasmid. The pellet was dissolved into 250 µL of the 'Resuspension Solution' (containing RNase) and mixing was done through gentle pipetting. After this step Lysis Solution (250 µL) was added and tube was inverted approximately 4-5 times so that solution would become clear. In the next step, 'Neutralization Solution'(350 µL) was supplemented into it and tube was inverted appoximately 4-5 times so that it became cloudy. Centrifugation was carried out at 14000 rpm for 6 minutes to settle down any debris and supernatant was transferred to GeneJet spin columns. Columns werecentrifuged at maximum speed for 60 seconds and the flow-through was discarded. In the next step 'Wash Solution'(500 µL) was added to the column. Again centrifugation was carried out, flow-through was discarded and this step of washing was carried out twice with the same conditions. In the next step residual wash solution was removed by spinning empty column for 1 minute and columns were taken out and placed in new centrifuge tubes. In the next step to elude the DNA Elusion Buffer was put in the column and centrifuged at maximum speed for 2mins after incubating it for. The purified plasmid was kept at - 20 °C and the column was discarded.

3.7 Restriction digestion analysis

To confirm the presence of insert, restriction digestion was performed For this purpose digestion with *Eco*RI and *Bam*HI (Fermentas, USA)was carried out; as

pTZ57R/T vector (TA vector) contained sites on restriction enzymes. The reaction mixture included plasmid (5), restriction enzyme (5U), 1X buffer for each enzyme respectively making up the volume to 20 µL and incubating the mixture for approximately 2-3 hours at 37 °C. Digested products were run on the gel (1%).

3.8 Analysis of Plasmid DNA by gel electrophoresis

The purified plasmid was analysed by using 1% agarose gel.

3.9 Sequencing of Clone

The purified plasmid was sequenced by forward and reverse universal M13 primers at Virology lab of Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST, Islamabad by forward and reverse universal M13 primers. The sequence was subjected to BLAST homology search to confirm the extent of similarity with published HBx sequence on NCBI at both nucleotide and protein level.

3.10 Transfection studies:

The transfection studies were performed on HCC cell line (Huh7). Huh7 cells were transfected with pTZ57R/T-HBx vector (cloned vector) and null control (plasmid vector).

3.10.1 Revival of the Cryopreserved Cell Line:

Huh7 cells were taken from the Cell culture lab of ASAB, NUST stored and maintained at -80°C. The cryovials were thawed and cells were revived according to standard protocol. The cryovials containing the frozen cells (stored in liquid nitrogen) were taken and placed immediately into 37 °C water bath and thawed.The complete growth medium (DMEM) made by supplementing DMEM 1X (Gibco, US) with 10% FBS and 100X penicillin-streptomycin (pen-strep) (Gibco, US); was pre-warmed and added to centrifuge tubes containing the thawed cells.The cell suspension was centrifuged at 200 xg for 6 minutes.After centrifugation the supernatant was discarded carefully and the cells pellet were carefully resuspended in complete growth medium and again centrifuged for 5 mins at 200 xg.The supernatant was discarded and pellet was carefully resuspended in 500µL pre-warmed complete media DMEM Gibco, US) and carefully transferred to T-25 culture vessel having 4mL of complete pre-warmed DMEM (Gibco, US). The culture vessels were checked under the inverted light microscope under 20X resolution and placed in 37°C CO2 incubator (5%). The cells were monitored regularly for any contamination and confluency check. Media washing was done every 3rd day.

3.10.2 Sub-culturing/ Trypsinization of cells:

When the cultured cells became 60%-70% confluent, the adherent cells were trypsinized to transfer to larger culture vessels.According to manufacturer's protocol, the cells were subcultured by aspiring the spent cell culture media from the culture vessel in the laminar flow hood. Cells were washed with freshly prepared PBS solution (2 mL per 10 ^{cm3} culture surface area) without calcium and magnesium ions.Wash solution (PBS) was carefully added to the side of vessel to avoid disruption to the cells and the culture vessel was rocked gently back and forth 4-5 times.The wash solution was discarded and 1 mL of pre-warmed Trypsin-EDTA 1X (Gibco, US) was added so that it covers the monolayer of cells.The culture vessel was rocked gently to distribute the dissociation reagent on the monolayer of cells and incubated in 37°C Incubator for ~5 minutes. The cells were observed under inverted microscope to check for cells detachment. The cells detached were <90% so the incubation time was increased and placed in 37°C Incubator and taped gently to ease cells detachment. For complete detachment, less than 500µL Trypsin-EDTA 1X (Gibco, US) is added in culture flask and incubated for 3 minutes. Finally, scrapers were used to detach cells with brief and gentle scraping. The cells were >90% detached, the culture vessel was titled to drain the dissociation reagent and 2 mL of pre-warmed complete growth medium was added (twice the volume of dissociation reagent). The medium was distributed over the cells surface by pipetting several times. The cells were transferred to 15 mL falcon tubes and then centrifuged at 200 xg for 6 minutes. The cells were resuspended in minimal pre-warmed growth medium.

3.10.3 Optimized Transfection:

A day before transfection, cells (trypsinized to be in suspension) were transferred to 6 well plates (confluency should be 70-80% at the time of transfection) and were counted under inverted microscope (using hemocytometer).Before transfection concentration and OD of DNA (plasmid-DNA) was checked at nanodrop, so the samples showing concentration greater than 80 ng and OD at 260/20 greater than 1.7 and less than 1.9 were used for transfection purpose. Lipofectamine reagent (Invitrogen, USA) was used for transfection purpose. For transient transfection manufacturers protocol for 6 well plate was used, and optimized by changing the amount of DNA and lipofectamine. At day 1, for transfection firstly 20µL of Plasmid-DNA (100ng/µL) and 20µL plasmid (null control) was added in two separate tubes containing 250µL of opti-MEM Reduced Serum

Medium in each tube respectively. In the 3rd tube 10µL (four amounts of it were used and got optimized to be 10µL) of Lipofectamine Reagent (Invitrogen, USA) was diluted in 250µL of opti-MEM Reduced Serum Medium (prepared separately for each sample and control vector) and incubated at temperature for 20 minutes. After incubation 250µL of Lipofectamine solution was mixed with diluted DNA solution and in another tube 250µL of Lipofectamine solution was mixed with diluted plasmid vector (null control). Incubation was again done for 5 minutes. The medium of cells was already changed before transfection with 2mL of complete DMEM (Gibco, USA). After incubation, the transfection medium of 500µL of control vector (pTZ57R/T) and 500µL of DNA (pTZ57R/T--DNA) were transferred to each well of 6 well plates and marked respectively. After this step, the cells in 6 well plates were placed at 37°C in CO_2 incubator for 24-48 hours. At day 2, cells were examined under inverted microscope for cell viability and cytotoxicity. At day 3 after 48 hours the cells after transfection was ready to be counted and to extract RNA for further analysis.

3.10.4 RNA Isolation and cDNA Synthesis:

3.10.4.1 RNA Isolation:

For RNA isolation from cells, standard TRIzol method was employed. The media was aspirated from the cells and the cells were washed with 1-2 mL of PBS. PBS was then removed from the cells and 1 mL of TRIzol was added to the cells. The plate containing cells was gently and briefly scrapped and the cell lysate (containing TRIzol) was removed from the plate, transferred into eppendorf tubes (1.5 mL) and incubated at room temperature for~ 5 minutes. Approximately 250µL of chloroform was added in each tube with vigorous shaking for ~ 15 seconds and incubated for 5 minutes at room temperature. Centrifugation at 10,000 rpm for 15 minutes at 4°C. was done. At this stage, there appeared 3 layers in each tube the top layer was clear and aqueous, middle layer was white precipitation (DNA) and bottom layer was pink coloured organic phase.

With the help of 1000µL pipette the top aqueous and clear layer was withdrawn carefully and placed in another 1.5mL eppendorf tube. In this tube ~550µL of isopropanol was added and mixed gently by shaking tube gently and incubated at room temperature for 5 minutes. After incubation, again centrifugation was carried out at maximal speed (14000 rpm) for 20 mins at 4°C. After centrifugation, the samples were directly placed on ice and at this stage the pallet was hardly visible at the base. Isopropanol was discarded out of the

tube and ~ 1 mL of 75% Ethanol was added to each tube and mixed gently. Centrifugation was again carried out at 9,500 rpm for 5 minutes at 4°C, ethanol was discarded and pellet was air dried (not too dry). After this ~ 15-25µL of DEPC water was added to RNA pellet and then the absorbance was measured at 260 nm was measured using nanodrop. So the RNA's having 260/280 value greater than 1.8 was selected for gene specific cDNA synthesis, lower values (< 1.8) indicates contamination or RNA is partially degraded; concentration of isolated RNAs were also measured.

3.10.4.2: cDNA Synthesis:

From the isolated RNA (s), cDNA was synthesized using cDNA synthesis kit (Thermo Fisher). The OD and concentration of RNA(s) was measured using nanodrop technology.Those RNA(s) were selected whose OD at 260/280 was greater than 1.8 (OD's of RNA were : 1.87, 1.88, 1.96,2.0)and respective concentrations (299.8ng/µL, 302.0ng/µL, 337.5ng/µL, 35.2ng/µL).After nanodrop readings gene-specific cDNA was synthesized using cDNA synthesis kit and reverse primer (DR) of the gene.As it was gene specific cDNA synthesis so instead of oligo dT, reverse primer for HBx gene was used. The reaction mixture was made according to the protocol and amounts listed in table 3.3, final reaction volume of 20µL was prepared and centrifuged (vortex and spin). For gene-specific primed cDNA synthesis, incubation was done at 42°C. for 60 minutes and the reaction was terminated by heating at 70°C for 5 minutes. After this reaction mixture (cDNA) was centrifuged (vortex and spin) and proceeded with PCR amplification. According to manufacturer's protocol reaction mixture was made as 5 µL of RNA, 1µL Primer (Reverse DR), 4 µL of RT buffer (5X), 0.5 µL RiboLock RNase Inhibitor(20U/µl), 2 µL of 10mm dNTP mix, 1 µL RevertAid RT (200U/µL) and 6.5µL, nuclease free water making total volume of reaction mixture upto 20 µL.

3.10.5 PCR for amplification and detection:

The cDNA (HBx) was amplified by Polymerase Chain Reaction. The reaction mixture and the temperature protocols followed are aforementioned.

3.10.6 PCR product analysis by gel electrophoresis

The products obtained as a result of PCR were run on the gel (1.2% agarose) which was prepared according to the protocol aforementioned.

CHAPTER 4: RESULTS

4.1 Sample Collection

Patient's positive for chronic HBV infection with the onset of liver disease and high viral titre were included in the sampling process. Total of 90 patients were included in the process of sampling .Data related to the patients on the whole shows 63 male patients and 27 female patients. The male patients were in the age range of 17 years to 71 years and female patients are 15 years to 70 years in age. The patients data specified their location to Abbottabad, Chakwal, Haripur, Islamabad, Lahore, Rawalpindi and Taxila with viral titre ranging from <3.0 to 937600.Furthermore the data collected from individuals (patients) were based on their approved consent

4.2 Detection of Viral DNA through Nested Polymerase Chain Reaction

Results of some of PCR samples after the final round of Nested PCR, appearance of 200 bp band on gel confirmed that those samples were positive and contained HBV genome are shown in Figure 4.1 and 4.2, respectively.

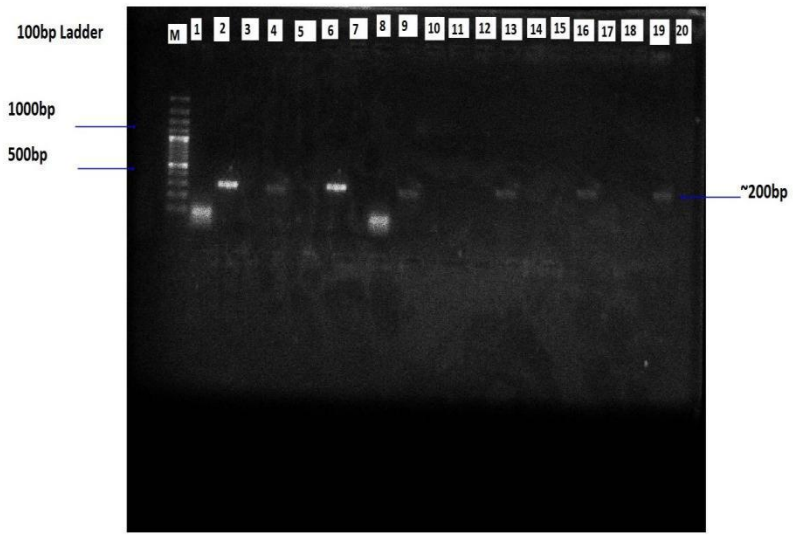

Figure 4.1: Detection of viral DNA through amplification of HBV core region by Nested PCR.Lane M:100 bp ladder; Lane 1-20:PCR products (Lane 2, 4, 6, 9,13, 14,16 and 19 indicate positive results for HBV DNA);Lane 20: Negative control

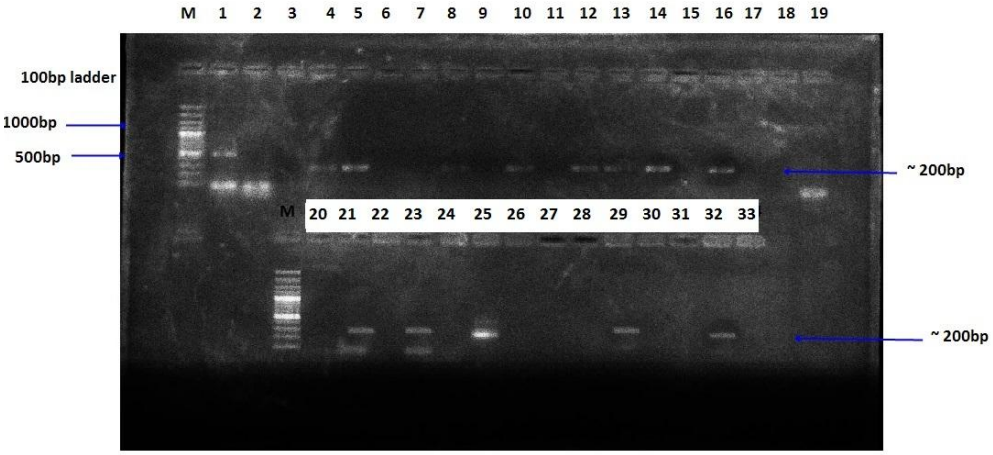

Figure 4.2: Detection of viral DNA through amplification of HBV core region by Nested PCR. LaneM:100 bp ladder; Lane1-33: PCR amplification from different samples(Lane4,5,8,10,12,13,14,16,21,23,25,29and 32 are positive for HBV DNA);Lane 20: Negative control.

4.3 Amplification of HBx Gene by PCR and Screening of Samples

Using the PCR profile for the amplification of HBx, the HBV positive samples were amplified and the PCR products were run on gel and the appearance of a band of approximately 465 bp confirmed the amplification of HBx gene in the samples. Figure 4.3 and 4.4 show some of the positive results.

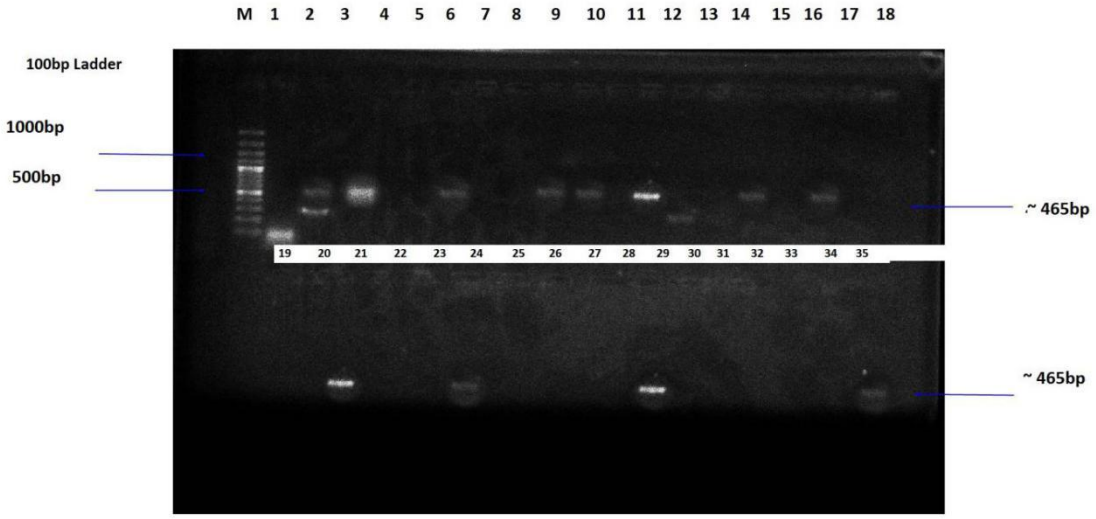

Figure 4.3: ScreeningofHBxgenefromviralDNAisolatedfrom the samples. LaneM: 100 bp ladder; Lane 1-35:PCR products(Sample 2, 3, 6, 9, 10, 11, 14,16,24,29 and 35 are positive for HBx gene); Lane 19 in lower wells :Negative control

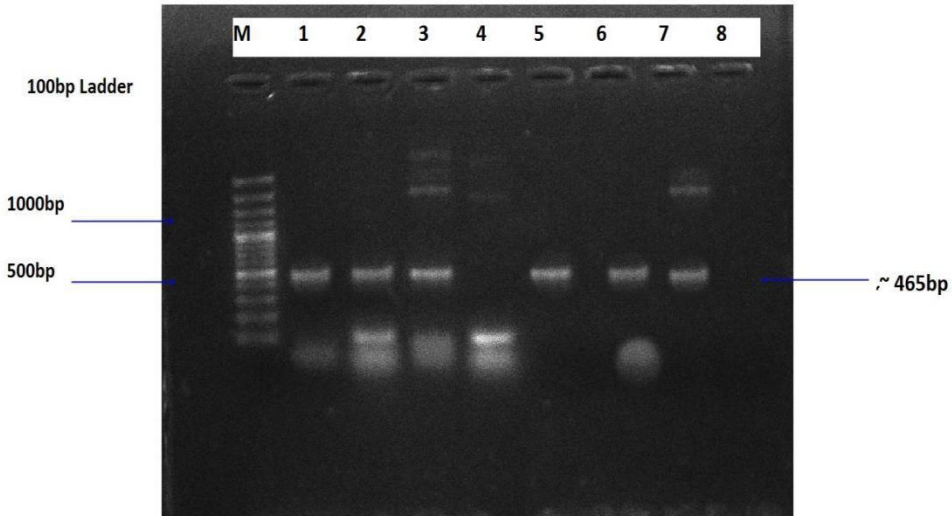

Figure 4.4: Screening of HBx gene from viral DNA isolated from the samples

Lane M: 100 bp ladder (Thermo Fisher Scientific,USA); Lane 1-8: PCR products (Sample 1 , 2, 3, 5, 6 and 7 are positive for HBx gene); Lane 8: Negative control

4.4 Cloning of HBx gene

4.4.1 TA vector Ligation

Cloning was done using one of purified gene products and TA vector.

4.4.2 Clone confirmation

4.4.2.1 Restriction digestion analysis

The Plasmid was treated with the restriction enzymes i.e EcoRI and BamHI (double digestion)producing bands size of approximately 2.8kb, 414bp and 50bp as indicated by Figure 4.5

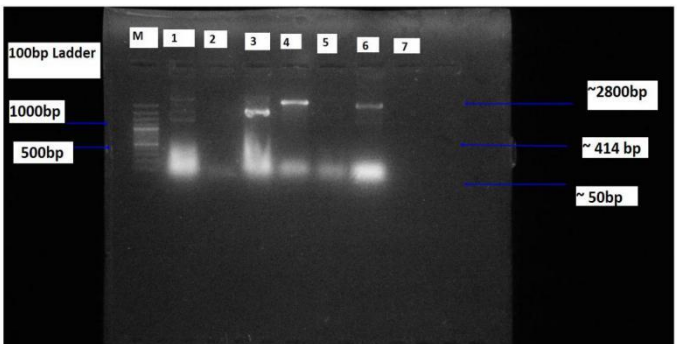

Figure 4.6: Confirmation of presence and orientation of clone through restriction digestion of pTZ57R/T--HBx clone by EcoRI and BamH1 (Double digestion).

Lane M: Ladder; Lane 1-7: Digestion of each of the seven samples on the gel as untreated plasmid, treatment with EcoRI and BamH1 (Double digestion). Appearance of ~500bp (414 bp) band, ~50 bp band and around 2800 bp band with EcoRI and BamH1 (Double Digestion) shows the confirmation of cloning

4.5 Plasmid Isolation

The positive clones of gene of interest were isolated and purified for the purpose of sequencing and analysed on gel. Figure 4.6 shows the purified plasmids containing our gene of interest.

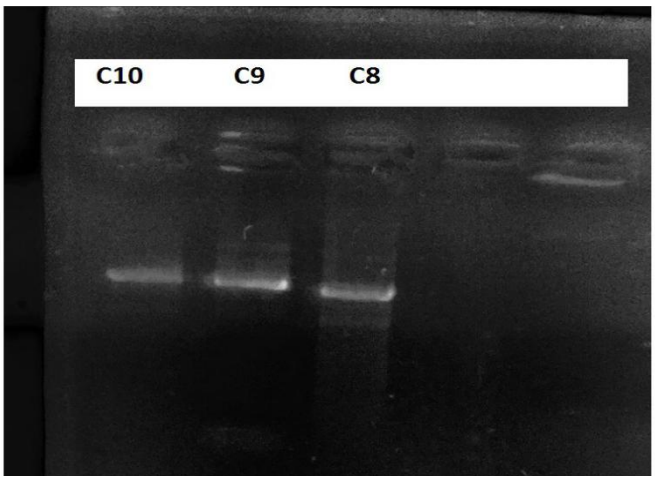

Figure 4.6: Isolated Plasmids: Purified plasmids containing gene of interest

4.6 Sequencing of Clone

The TA vector cloned with HBx (our gene of interest) was sequenced at Virology Lab of Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST by using both forward and reverse universal primers (M13) and the results were subjected to BLAST Homology search at NCBI. The results depicted 93% homology of the sequence with published HBx gene sequence. The nucleotide sequence was translated into amino acid sequence using ExPasy Translate tool and subjected to BLAST Homology search for proteins. The results depicted 90% homology with published HBx protein sequence.

4.7 Revival of the Cryopreserved Cell Line and the Transfection studies:

The cells were revived and grown using optimal growth conditions.The transfection studies were performed on HCC cell line (Huh7). Huh7 cells were transfected with pTZ57R/T-HBx vector (cloned vector) and null control (plasmid vector).Figure (A) (i) the cells at 60-70% confluency before the transfection, (ii) shows the control cells after

48 hours of transfection, (iii) shows the experimental cells (plasimd-DNA) after 48 hours of transfection respectively.The cells were observed under inverted microscope at 10X resolution

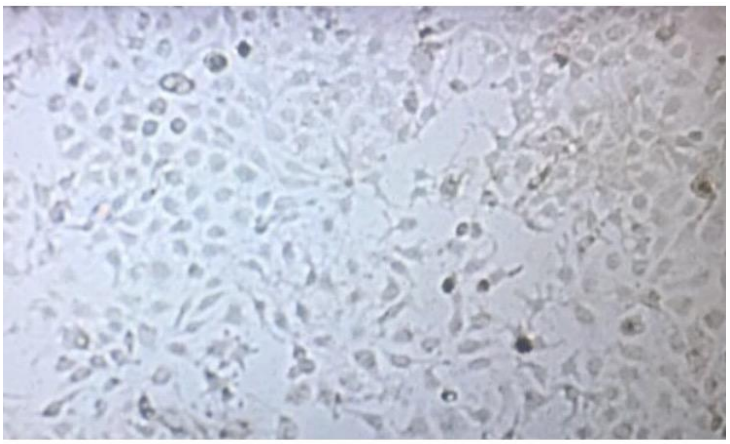

Figure (A) (i) the cells at 60-70% confluency before the transfection at10X

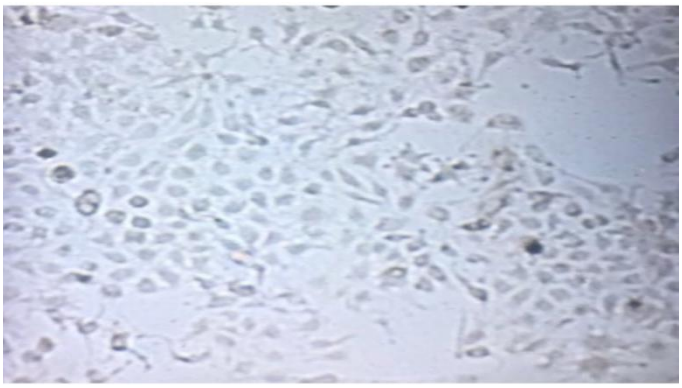

Figure (A) (ii) shows the control cells after 48 hours of transfection at 10X

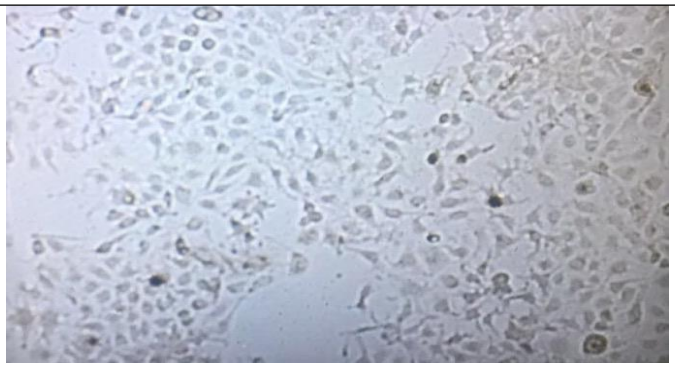

Figure (A) (iii) shows the experimental cells (plasimd-DNA) after 48 hours of transfection at 10X

4.8 RNA Isolation from Transfected HCC cells and cDNA Synthesis:

RNA was isolated from transfected cells and cDNA was synthesized to be used for further experiments.

4.9 PCR for Amplification and Detection:

The cDNA (of HBx) was amplified by Polymerase Chain Reaction by using the optimized PCR protocol aforementioned. The PCR products were run on gel and visualised under UV transilluminator and photographed in gel documentation system. The figure 4.12 shows the presence of bands of ~465bp as compared to null control (plasmid vector).

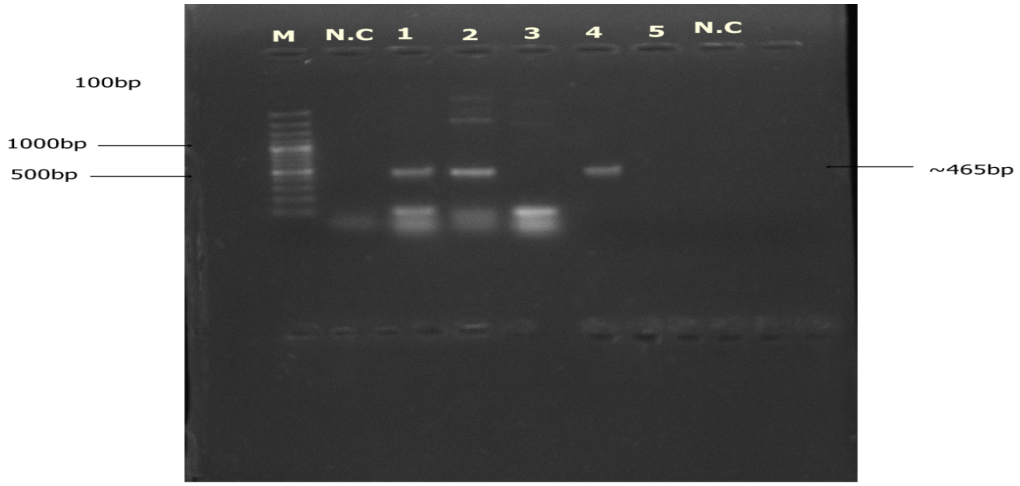

Figure 4.12: Positive bands of cDNA (~465bp) amplified by PCR

Figure 4.10: PCR results of HBx amplification. Lane N.C (are negative controls of PCR and Transfection), Lane 1,2 and 4 shows positive bands of ~465bp.

CHAPTER 5: DISCUSSION

The carcinogenesis of hepatic cells, known as hepatocellular carcinoma is actually the malignancy of liver being caused by chronic infection with HBV (one of the attributor) (Bréchot*et al.,* 2000). The transactivating role of hepatitis B virus is due to HBx gene/protein. Hepatitis B virus X protein affects major cellular pathways as well as cell cycle and apoptosis linked to cells by making different interactions with number of proteins in liver cells which are usually acute or chronically infected with virus. Thus HBx has central role in HBV induced hepatocellular carcinoma.(Xiang & Liu, 2010). It has been shown recently that one of the mechanism by which HBx protein attenuates cellular transcription by changing the transcription of methyl transferases therefore resulting into hypermethylation (regionally and globally) resulting into gene silencing such as tumor suppressor gene or instability of chromosomes and in this way results in carcinogenesis of hepatic cells (Kew, 2011).

The Hepatitis B virus x gene can be used to transfect human HCC cells and it can be transcribed in them because it can easily incorporate in the host genome, although it can do this function even in the absence of replication (Hwang *et al.,* 2003; Peng *et al.,* 2005). The notion that HBx is important in the life cycle of virus has yet not been clearly explained but studies on woodchucks shows that HBx is really important for infection to occur in WHV. (Chen *et al.,* 1993; Zoulim *et al.,* 1994). There is observation that HBx is actually involved in transactivation of such genes which are in charge of regulation of growth of cell so it suggests that HBx may stimulate uncontrollable cell growth (Yen, 1996). Our study aims to show the role of hepatitis B virus X protein in the development of HCC. In a study it has been observed that HBx is involved in stimulating the growth in inactive fibroblast cells (Koike *et al.,* 1994), and it is also involved in de-controlling the checkpoints of cell cycle (Benn and Schneider, 1995).

In an experimental study which was conducted to find the role of X mutants on the proliferation suggested that abolition of effects of apoptosis and anti-proliferation of hepatitis B virus X protein by mutations that are present naturally might make the hepatic cells prone to indefinite cell growth and as result can lead to carcinogenesis in hepatocytes which is linked to infection caused by Hepatitis B virus (Sirma *et al*., 1999). In our study HBx was successfully transfected on HCC cells

In the recent studies it has been shown that proliferative activity of HCC cells is due to HBx, therefore an experimental study shows that growth pattern of cells were determined which were excessively expressing HBx and it is shown that Huh 7 increases in growth and number when they were transfected with HBx plasmid as compared to Huh 7 cells which were transfected with null vector (control plasmid) (Damania *et al.,* 2014).

In another study, it was concluded that transfection studies done on Huh7 cells using hepatitis B virus X protein (mutants), differential kind of expression was noted. Also it was observed that those gene and proteins whose expression was differential. Have role in carcinogenesis, cellular signalling. regulation of transcription and responses related to immune system. Thus, supporting the notion that HBx (mutants) is involved.in hepatocarcinogenesis (Xiaohong Liu *et al.,* 2008).

In our study transfection was carried out on Huh7 cells using plasmid HBx (clone) and visible difference was noted on the proliferation of Huh7 HCC cells showing that HBx is involved in proliferation of HCC cells which was further supported by the amplification of cDNA (from RNA extraction).

Hepatitis B Virus X protein being only hepatitis B virus protein expressed in malignant hepatocellular carcinoma and it has crucial role in pathogenesis of hepatic carcinogenesis. Although the exact process by which HBx induces oncogenesis is still obscure but evidences supported the notion that it clearly plays pivotal role in carcinogenesis of hepatic cells (chronically infected) (Geng, & Liu, 2015). Our study also outlined this fact that HBx is critically involved in carcinogenesis of hepatic cells taking in account the HCC cells and comparing the results of HBx transfected Huh7 cells and Huh7 cells which were transfected with null control (plasmid vector).

The mechanism behind how HBx is causing carcinogenesis is well explained by its function in interaction between the proteins, causing instability in chromosomes, translocations, transcription factors regulation, involvement in cellular signalling and in this way HBx has role in cell growth, and malignant transformation of cells.

In a study, conducted on Huh7 cell line to observe that Hepatitis B virus X protein is affecting the expression of gene making in use of HCC cell. Line (Huh7) that how Hepatitis B virus X protein is playing role in carcinogenesis of chronically infected hepatic cells. The expression of Hepatitis B virus X protein was observed and confirmed

in HCC cells and was carried out through microarray analysis. It was shown that some of the genes were comparatively upregulated and further in some genes their expression drops down (Zheng *et al.,* 2009) but conclusively this study matches the results of our study that HBx was significantly expressed in HCC cell line and has important role in carcinogenesis of hepatocytes.

In another experimental setup transfection studies were carried out with a control plasmid (pEG plasmid) and experimental HBx gene. The transfection was carried out in HCC cell lines Huh7 and HepG2 to check the expression hepatitis B Virus X protein. After transfection in these cells gene expression was measured. There was stable expression of HBx mRNA in Huh7 and HepG2 cells, so the study clearly showed the expression of peG-HBx expression in cells being transfected as compared to cells being transfected by null control (plasmid), (Park *et al.,* 2011) similarly in our study there was enhanced expression of mRNA of HBx protein as confirmed by the amplification carried out through PCR.

Hepatitis B virus X protein has shown high level of expression in HCC cell lines and HBx induces proliferation through the miRNA-21 is one of the mechanism and it was carried out in a study showing the high expression of hepatitis B virus X protein. In this setup transfection efficiency in HCC cell lines such as HepG2 and Huh7 cells and is observed to be greater than 80% as compared to control vector. It was found that expression of Hepatitis B virus X protein was comparatively high in transfected cells as compared to control vector transfection and was assessed through different assays (Diamantis *et al.,* 1992), as similar results were indicated by our study showing high proliferative effects on Huh7 cells transfected by HBx gene (pTZ57R/T-HBx) as compared to control vector (pTZ57R/T.

Conclusively, the experiment was successful in amplifying HBx gene from selected patients and the HBx gene (cloned in plasmid) was successfully transfected in HCC cell line.

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