Epigenetic analysis of hepatocellular carcinoma development : involvement of HBV-modulated DNA methylation

Niu, Dandan

2010

Niu, D. D. (2010). Epigenetic analysis of hepatocellular carcinoma development : involvement of HBV-modulated DNA methylation. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/40634>

<https://doi.org/10.32657/10356/40634>

EPIGENETIC ANALYSIS OF HEPATOCELLULAR CARCINOMA DEVELOPMENT: INVOLVEMENT OF HBV-MODULATED DNA METHYLATION

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A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

2010

Acknowledgement

Looking back of the three years of my PHD life, what I want to express are all my appreciations.

First, I would like to express my sincerest thanks to my supervisor Associate Professor Chen Wei Ning, for his precious guide and consistent supports. Throughout my PHD program, Prof. Chen gave me all his trust and encouraged me to try my own ideas. He could always get me out when I encountered problems and got frustrated by failure of the experiments. I really appreciated the atmosphere my supervisor created for me: the freedom in performing research enabled me to work creatively and independently; while his timely advices and suggestions helped me to overcome every difficulty and achieve advances. In addition to the studies, I also want to thank Prof. Chen for his great example of a healthy balance of work and life, and his understanding and encouragement on my pursuing other interests in my life. All the help and kindness from Prof. Chen makes me feel lucky and are deeply appreciated!

It's also very happy to work with all my previous and present colleagues. Thanks to Dr. Tan Tuan Lin for his help with my experiments and teaching me a lot of useful techniques; thanks to Dr. Feng Huixing for cooperation and helpful discussion in the design of experiments and techniques; thanks to Ms. Ren Yudan for help with the FACS assay and discussion on the results; thanks to Dr. Pan Hong, Dr. Lu Yiwei, Dr. Sui Jianjun, Mr. Zhang Jianhua and Ms. Bai Jing, for their valuable discussions and supports with my

experiments; thanks to Ms. Zhou Yusi for help with the literature study; thanks to Mr. Wang Mingxuan Ms. Bahareh Haji Rasouliha and Ms. Laleh Sadrolodabae for their kind supports.

Thanks to my dear friend Ms. Qiao Yan for help in statistical analysis and IT supports; thanks to Mr. Lu Zhisong, Ms. Li Rui for care and help in both courses and daily life; thanks Dr. Li Jianliang for discussion on epigenetics; thanks to Mr. He Hujun for help in searching literatures. Thanks for all their friendship and supports!

And special thanks to Mr. Li Xiang, for his love and care all the way since we met each other.

Warmest thanks to my family who are always there for me to turn to. Without the love and trust from them, I cannot be a confident and healthy person like today. I want to present this thesis to my beloved parents, for their most selfless love and care, for their understanding and support to my choices all the time.

Last but not least, thanks to my university and school, Nanyang Technological University, School of Chemical and Biomedical Engineering, for providing me with not only scholarship, but also facilities and services, and all kinds of supports for my research.

Thank you all! Thank you so much!!

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Summary

Hepatocellular carcinoma (HCC) is one of the most aggressive human malignancies. Epidemiological studies have shown that HCC is closely associated with chronic hepatitis B virus (HBV). However, the underlying mechanisms of the HBV-associated HCC development remain unclear.

DNA methylation, one of the mechanisms in epigenetics, affects gene expression at the transcriptional level. Evidence has revealed the association between HCC and HBV-mediated DNA methylation. Within the four proteins encoded by HBV genome, the HBV X protein (HBx) has been found to epigenetically inhibit some tumor suppressor genes (TSGs), which may lead to development of HCC.

To investigate comprehensively the role of HBV/HBx in DNA methylation, a protein profile affected by HBV in HBV-producing HepG2.2.15 cells compared with HepG2, was analyzed by our iTRAQ-coupled 2-D LC/MS-MS analysis. Fifteen proteins including S100A6 and ANXA2 were identified. A role of these proteins as target of HBV-mediated DNA methylation was supported by validation assays, including their re-activation in cells treated with 5-Aza-dC (a DNA methyltransferase inhibitor). DNA methylation analysis suggested that, the proteins were down-regulated by HBV via DNA hypermethylation.

In addition to the analysis of protein profile regulated by HBV-mediated DNA

methylation, the role of HBx was also analyzed. The HBx-induced protein profile in HepG2 was established by LC-MS/MS analysis. Interestingly, a number of the proteins were also identified between HBx-transfected HepG2 and HBV-expressing HepG2.2.15. Decreased expression of some genes has been linked to cancer development, suggesting their function as TSGs. Combined our results with other published investigations, two TSGs were selected for further mechanism and functional studies on HBx-mediated DNA methylation in HCC development.

One is glutathione S-transferase P1 (GSTP1). GSTP1 is a detoxification enzyme and has been considered as a TSG (frequently repressed/silenced in human cancers including HCC). Promoter hypermethylation results in its silence, and has been associated with HBV-associated HCC in clinical observations. We found that GSTP1 expression was depleted in HBV-producing HepG2.2.15 as a result of the DNA hypermethylation. Significantly, HBx genotype D was found to repress GSTP1, which was confirmed by further functional studies including Reactive Oxygen Species (ROS) and apoptosis analysis.

To further validate and confirm the role of HBx in epigenetic regulation, Deleted in Lung and Esophageal Cancer 1 (DLEC1) was analyzed. DLEC1 is another TSG frequently silenced by DNA hypermethylation and histone hypoacetylation in its promoter in human cancers including HCC. However, there is no evidence in the link of epigenetic silence of DLEC1 to HBV. We found that HBx genotype A down-regulated DNA methylation and up-regulated histone acetylation, resulting in an increased DLEC1

expression. Taken together of GSTP1 and DLEC1, our data suggested that HBx was implicated in the regulation of TSGs in a genotype-specific manner and this provided new insights in the impact of HBV genotypes on the epigenetic modulations in HBV-associated HCC development.

In conclusion, we have established a new proteomic platform to analyze comprehensively cellular protein profile associated with epigenetic modifications. The identified proteins could well be candidate targets of HBV/HBx-mediated DNA methylation. Additional functional studies on two TSGs revealed the direct involvement of HBx in the regulation of the TSGs. It is to be hoped that further comprehensive analysis of target genes identified from our approach should contribute to effective antiviral strategy, leading to a better control of HBV-associated HCC.

Chapter 1 Introduction

1.1 Introduction

1.1.1 HCC and HBV

HCC has been considered as the most frequent primary solid tumor of liver and one of the most aggressive malignancies in human. Not less than 550,000 new patients are diagnosed with HCC worldwide each year [3, 4]. Although it is numbered as the fifth cancer in occurrence, it is ranked as the third highest in mortality due to its poor prognosis [5]. There is significant geographic variation in its occurrence all over the world. It is most prevalent in most part of sub-Saharan Africa and Asia, especially China. In most of western countries such as Europe and North America, the incidence is low [5-7]. However, a rising incidence of HCC in UK and USA has been observed in the last two decades [8, 9]. Much effort has been investigated to improve the diagnosis as well as treatment of HCC, but the therapeutic results remain unsatisfactory.

Although other risk factors were identified including dietary exposure to aflatoxin B₁, alcohol abuse, hemochromatosis, fatty liver disease, androgenic steroid use and α -1 antitrypsin deficiency [10], evidence indicates that HBV is one of the major etiological factors leading to HCC development. HBV is found to be the etiologic agent for 75%-90% of the primary liver cancers [6, 11]. Epidemiological reports have revealed that high prevalence of HBV infection is linked to high rates of HCC (Fig. 1.1) [12]. Despite

these reports, there is still lack of understanding on the underlying mechanisms of the HBV-associated HCC.

Figure 1.1 Worldwide distribution of chronic HBV infection and annual incidence of primary HCC. The darker blue indicates areas with higher prevalence of HBs Ag carriers, while darker red indicates areas with higher incidence of HCC. HBs Ag = hepatitis B virus surface antigen. [12]

1.1.2 Organization of HBV and the role of HBx

HBV is a circular, partially double-strained DNA virus. It is highly organized within a compact genome of approximate 3200 base pairs, with slight differences in sequences among genotypes [13, 14]. The HBV genome encodes four overlapping open-reading frames (ORF) including PreS/S, PreC/C, P and X proteins (HBx), as shown in Fig. 1.2 [15]. HBV is believed to integrate randomly into its host genome and this integration may activate adjacent cellular oncogenes which will contribute to HCC development [16]. Particularly, the integrated HBx appears to be the most frequent viral marker in HCC [17, 18].

Figure 1.2 Organization of HBV genome. The inner circles represent the circular partial double-stranded HBV genome. HBV polymerase (orange circle) is for viral synthesis. Numbering of nucleotide is based on the site of unique restriction enzyme EcoRI (position 1). The different open reading frames including S, core, polymerase, and X, are shown by arrows. Abbreviations: S, surface antigen; Y, tyrosine; M, methionine; D, aspartate; DR, direct repeat segment. [15]

The small HBx protein consists of 154 amino acids. It is not a structural protein, but a very versatile regulatory element involved in numerous cellular activities and signal pathways. These include transcriptional regulation, cell cycle, cell proliferation, DNA repair, cell adhesion, and apoptosis [14]. HBx has thus been considered to be important not only essential for virus infection and integration into host genome, but also involved in hepatocellular carcinogenesis [19]. Recently, its function has been extended to the field of epigenetics, with a role in modulating DNA methylation via regulating the expression of DNMTs [20-23].

1.1.3 Genotypes of HBV and HBx

HBV has been categorized into 8 genotypes (A-H) based on DNA sequence divergence [24, 25]. Among the different genotypes, there are not only geographic and ethnic distribution variations (Fig. 1.3) [26], but also clinical and pathogenic differences [27-29]. Recent evidences have also pointed to an association of HBV genotypes characterized by their distinct geographic distribution with distinct clinic manifestations. For example, cirrhosis and HCC are more frequent in carriers of genotype C than B [30-33]. However, molecular and virologic mechanisms underlying the clinical phenotypes of different HBV genotypes have been poorly understood.

Figure 1.3 Geographic distribution of HBV genotypes. But existing information is incomplete, as data in many parts of the world are not available or are based on very small numbers of patients studied. Additionally, the distribution of HBV genotypes may vary with time and with population migration [34]. Furthermore, subtypes have now been identified within some genotypes. [26]

Similarly to HBV genome, HBx has also been categorized into 8 genotypes. Because in each genotype of HBV genome, there are some sequence variations in its HBx region. Therefore, HBx genotype A indicates the HBx region within HBV genotype A, and similarly we have HBx genotypes B-H which are from HBV genotypes B-H, respectively. S. Datta. and coworkers reported that genotype-associated clinically HBx variations could evolve and act distinctly in different geo-ethnic populations [35]. Furthermore, our recent investigation has indicated that the analysis of cellular proteins associated with genotype-specific HBx may be helpful for identifying early diagnostic markers for HCC [36].

1.1.4 DNA methylation in cancers including HCC

Today, a wide variety of illnesses including cancers have already been linked with epigenetic modulations via, for example, abnormal DNA methylation.

DNA methylation is the only form of naturally occurring DNA modification. It refers to the conversion of cytosine into 5-methylcytosine in the CpG dinucleotides located within gene promoter region [37]. Once the promoter is methylated, a series of down-stream events may take place, resulting in the expression of the gene being inhibited or even totally silenced. If for example, the promoter of a tumor suppressor gene (TSG) is methylated, the reduced or absent of TSG expression would occur and this may lead to cancer development.

Cancer cells are usually characterized by global genomic hypomethylation, and focal hypermethylation of CpG islands of TSGs which are generally unmethylated in normal cells. The hypermethylation and silencing of TSGs directly impact on carcinogenesis. The potential CpG islands which are target of DNA methylation are usually located near the transcription start sites of TSGs. Hypermethylation of these CpG islands usually leads to the inhibition of expression of down-stream genes, as indicated in Fig. 1.4. This has been one of the most common mechanisms of inactivation of TSGs in cancers [37].

Figure 1.4 Establishment and reversion of epigenetic mutations. (A) In healthy cells, tumor suppressor genes are unmethylated and expressed at normal levels. (B) In cancer cells, hypermethylation of the promoter (and/or exon 1) region leads to gene silencing. (C) Treatment with DNA methyltransferase inhibitors (DNMT inhibitors) removes the majority of 5-methylcytosine ($-CH_3$), and can reactivate gene expression. [38]

Hypermethylation of TSGs has been considered as one of the most vital hallmarks of human cancers, including primary liver cancer HCC. Aberrant DNA methylation of a number of genes has been found to arise very early, even in histologically normal liver from HCC patients [39]. Therefore, the DNA methylation status of target gene has been proposed to be a kind of promising biomarker, in addition to identification of differentially expressed proteins in cancers as biomarkers [40-43].

There are at least two advantages of using DNA methylation as a biomarker. The first

is that aberrant DNA methylation can occur in the very early stage of cancer development and therefore could provide important information during early detection and diagnosis in populations with high risk of developing cancers. For example, aberrant methylation of p16^{INK4a} has been considered as an early event in lung cancer and a potential biomarker for early diagnosis [44, 45]. It has been successfully assessed to be used for early detection for bladder cancer [46]. In addition, evidence has suggested that aberrant methylation of RASSF1A (Ras association domain family member 1) is an early event in the development of HCC [47]. The second advantage is that non-invasive detection techniques have been developed with higher sensitivity and specificity, such that sputum, serum/plasma [48-51] or urine [52, 53] can be used.

In the case of HCC, it has been known that aberrant DNA methylation is an important epigenetic alteration in its development. Several comprehensive methylation studies have established the methylation profiles of a number of TSGs in aging liver, chronic hepatitis and HCC. These profiles indicate that many of them are usually hypermethylated in HCC [54-56]. For instance, significant link has been reported between the hepatocellular carcinogenesis and the silence by DNA hypermethylation of several TSGs, like IGFBP-3, RASSF1A [22], p16^{INK4a} [57], p15 [58], E-cadherin [59, 60], GSTP1 [61] and DLEC1 [62]. And among them, RASSF1A is hypermethylated in the early phase of HCC while p16^{INK4A} is hypermethylated in the late phase of HCC [63].

1.1.5 DNA hypermethylation and HBV-associated HCC

The involvement of HBV in HCC development has been implied by numerous clinical and laboratory studies [64, 65], and extensive reports have suggested different mechanisms involved in HBV-associated HCC [66, 67]. However, the first few reports uncovering aberrant epigenetic modifications of target genes in hepatocarcinogenesis induced by HBV have only surfaced in the past few years. It is believed that persistent inflammation caused by virus or viral genome integration in the host genome could contribute to aberrant epigenetic events such as DNA methylation [68, 69]. Recently, one of the four proteins encoded by HBV genome, HBx, has been implicated in epigenetic regulation of TSGs [20, 59]. The involvement of HBx in global hypomethylation and regional hypermethylation of target promoters involving deregulation of DNMTs [22], has resulted in the silencing of TSGs and the development of HCC. Most of the genes which have been found to be aberrantly methylated in HCC, like p15, p16, [20, 70-72] E-cadherin [21, 59], IGFBP-3 [22], have been associated with HBV or HBx. A proposed model for the DNMT1 regulatory circuit and its activation by HBx is summarized in Fig. 1.5 [20].

Figure 1.5 Target genes regulated by HBx via DNMTs. “ ” indicates down-regulation, while “ ” indicates up-regulation. The direct interaction of HBx and DNMT3A leads to two distinct effects of the down-stream genes, up-regulation of IGFBP-3 and CDH6, but down-regulation of IL-4R and Metallothionein-1F. DNMT3A was also reported to inhibit IGFBP-3 expression, but the mechanism was not determined. [2, 20-23, 71]

1.2 Motivations

Hepatocellular carcinoma (HCC) is the most common primary liver cancer, which has high mortality rate due to poor prognosis. Extensive studies on hepatocarcinogenesis have been focused on numerous genetic abnormalities, including chromosomal deletions and rearrangements, aneuploidy, gene amplifications and mutations in tumor suppressor genes. Increasingly, the epigenetic mechanism of HCC development has received more attention

and an epigenetic model of the tumorigenesis has been established. In this model, genome wide hypomethylation results in increased chromosome instability, while regional hypermethylation decreases tumor suppressor gene expression and increases tumor risk [22]. HBV is one of the most significant causative agents of HCC. Clinical and molecular evidences have indicated that the HBV infection and replication results in DNA methylation alteration of the host genes. The abnormality of DNA methylation status of several genes, such as p16 [20, 70], E-cadherin [21, 59] and GSTP1 [61], has been associated with HBV infection. However, the exact targets and mechanisms of epigenetic abnormalities, especially of aberrant DNA methylation in HBV associated HCC, remain to be established.

Among the four proteins encoded by HBV genome, the multifunctional viral protein HBx has been analyzed for its role in epigenetics [20, 59]. The involvement of HBx in regional hypermethylation and genome wide hypomethylation of target promoters [22], has been suggested to result in the silencing of TSGs and leads to the development of HCC [21, 59].

However, the current understanding of the HBV-related HCC development as a result of epigenetic modifications has been limited by the overly focused approach. Specifically, only individual target genes have been analyzed so far. For example, p16 [20] and E-cadherin [21]. Such an approach may not provide comprehensive understanding on the epigenetic basis of development of HBV-associated HCC. Therefore we need to develop a profiling model to identify new targets of HBV/HBx and to enrich the understanding of

the role that HBx plays in epigenetic hepatocarcinogenesis. The identified proteins and their DNA methylation status both could be biomarkers for early diagnosis and even followed treatment of HBV-associated HCC.

The new target genes epigenetically modified by HBV, to be identified by our comprehensive analysis, would then be characterized further for their involvement in HCC development. Taken together, findings generated from our analysis should provide new information of HBV-associated HCC development, as a result of epigenetic modifications.

1.3 Objective of the project

The two overall objectives of the project are as follows:

1.3.1 Proteomic analysis of target proteins regulated by HBV via DNA methylation

- 1) To develop a cell-based proteomic platform for epigenetic analysis.
- 2) To identify new targets of HBV-associated DNA methylation as potential biomarkers for early diagnosis and treatment of HBV-associated HCC and therefore improve the understanding of the role that HBV plays in epigenetic hepatocarcinogenesis.

1.3.2 Case studies of TSGs epigenetically affected by HBV/HBx

- 1) To explore the molecular mechanisms of epigenetic regulations of target genes by HBV/HBx, especially via DNA methylation.
- 2) To study genotype-specificity of HBx functions on epigenetic regulation of TSGs, to provide new insights on the impact of HBV genotypes on the epigenetic modulations in HBV-mediated HCC development.

Chapter 2 Literature Review

2.1 Overview of DNA methylation and Epigenetics

2.1.1 Epigenetics and its mechanisms

The basis of the conventional genetics is that DNA sequence carries all the heritable information for protein expression; it is a cornerstone on which modern biology sits. However, several groundbreaking studies have recently revealed that gene function can be altered by more than just changes in DNA sequence. One of such alterations is referred to as epigenetics. It is a momentous shift in the understanding of inheritance.

The differences in protein synthesized with the same genome, come from the activation of some genes as well as inactivation of others. Actually most of the differentiation processes are triggered and maintained by epigenetic mechanisms [73]. Thus it is not just genes, but how they are regulated (the epigenetic control), that makes each protein and explains most of our phenotype variations.

Epigenetics is defined as the study of heritable changes in gene functions that do not involve changes to the nucleotide sequence of DNA. The changes are heritable, as they can be stably transmitted to subsequent generations through mitosis/meiosis. Major epigenetic mechanisms include DNA methylation, histone modification, RNA-mediated

phenomena [74, 75], and chromatin remodeling [76]. Among these, histone modification includes acetylation, methylation, phosphorylation and ubiquitylation [77, 78]. The posttranslational modification of histone proteins as well as DNA methylation at CpG sites, and the remodeling of chromatin (the complex of DNA and histone), are all involved in the way DNA is packaged. Other epigenetic changes are RNA involved, mediated different splice forms of RNA or double-stranded RNA [79]. Therefore, epigenetic modifications add an “extra” layer to the traditional transcriptional control of gene expression.

2.1.2 CpG islands and DNA methylation

DNA methylation, which is the only naturally occurring DNA modification and also one of the main mechanisms in epigenetics, affects gene expression and chromatin stability at the transcription level. It refers to the addition of a methyl group ($-\text{CH}_3$) to one of the bases that constitute DNA (in human, it is limited to cytosine), as shown in Fig. 2.1. DNA methylation is actively involved in various cellular processes. These include transcriptional regulation of gene expression, silencing of transposable elements, and the cellular defense against viral intrusion [75, 80, 81].

Figure 2.1 Sketch map of DNA methylation. The left panel shows the position of methyl group; the right panel indicates the methylated cytosine in DNA strain. (Image source: www.scq.ubc.ca/human-cloning-science-fiction-or-reality/)

DNA methylation status can be “inherited” during DNA replication. This transmission is catalyzed by a group of enzymes named DNA methyltransferases (DNMTs). The two main types of DNMTs include DNMT1 and DNMT3A & 3B. DNMT1 is the most active enzyme in catalyzing the post-replication methylation of DNA. By doing this, it is responsible for maintaining the promoter CpG methylation pattern from parental to daughter DNA stand during DNA replication [82]. On the other hand, DNMT3A and DNMT3B take part in the *de novo* DNA methylation during early embryogenesis [83, 84].

Like many other enzymes, DNMTs can be inhibited. DNMT inhibitors, such as 5-aza-2'-deoxycytidine (5-Aza-dC), directly repress tumor angiogenesis. This suggests that epigenetic modifications mediated by DNMTs are involved in regulation of gene expression during tumor angiogenesis [85]. 5-Aza-dC functions as a demethylation reagent, sequestering DNMT1 to 5-Aza-dC-substituted DNA via the irreversible binding of cysteine in the catalytic domain of DNMT1 [86, 87]. This results in a decrease of DNMT1 concentration in the cell and leads to genomic DNA demethylation during successive cell divisions [88-90].

In mammals including humans, normal DNA methylation is limited to one of the bases Cytosine (C) when it is followed by a Guanosine (G). Therefore, DNA methylation only takes place at CpG sites, where the "p" in “CpG” notation refers to the phosphodiester bond between the cytosine and the guanosine. It is estimated that in vertebrates, 60-90% of CpG sites within the genome are methylated [91, 92]. However,

this methylation appears primarily in areas with low CpG density [93]. Most CpG islands (with high CpG density) are unmethylated, especially in young people, and the level of methylation increases with age [94, 95]. Such small UNmethylated CpG clusters from the larger number of Methylated CpG sites in the whole genome, are referred to as CpG islands. Interestingly, most of the CpG islands are located in the promoter region of genes, and the changes in the methylation status play an important role in transcriptional regulation of their down-stream gene expression.

2.1.3 Regulation of gene transcription by DNA methylation and its biological significance

In general, reduction in methylation causes increase of gene expression, while increase of methylation leads to transcription inhibition or even silencing [81, 84]. As shown in Fig. 2.2A, when the CpG sites are unmethylated (green ellipses), the transcription factors (TFs, yellow circles) are able to bind to their target sites and allow gene transcription. In contrast, when CpG sites are methylated (red circles), the $-CH_3$ groups would block the binding of TFs and hence inhibit the transcription (Fig. 2.2B). Specific proteins are found to be involved in this process such as methylated CpG binding proteins (MBPs). MBPs are recruited to the methylated CpG sites to preclude the binding of TFs to the promoter region (Fig. 2.2C) [96].

Figure 2.2 Mechanisms by which DNA methylation inhibits gene transcription. (A) Hypomethylation of CpG dinucleotides within a promoter region allows transcription factors to bind and transcription to occur. (B) Methylation of CpGs inhibits transcription factor binding and therefore gene transcription. (C) The presence of MBPs bound to CpGs within a promoter region also blocks access of transcription factors to their binding sites [96].

The biological significance of DNA methylation has now been identified in several aspects and different species. For example, in mammals DNA methylation is very important in development and carcinogenesis.

In normal development, DNA methylation is essential for proper genomic imprinting. The paternal and maternal genomes are not equivalent and both are required for mammalian development [97]. The difference between the parental genomes is believed to be due to gamete-specific differential modification, known as genomic imprinting. The role of DNA methylation in genomic imprinting has been extensively reported in early 1990s [98-103]. The most convincing studies were about three imprinted genes, H19, insulin-like growth factor 2 (Igf-2) and Igf-2 receptor (Igf-2r), which are differentially

methyated depending on their parental origin [98-102]. A further examination of the three imprinted genes in mutant mice with deficient DNMT activity indicates that, the expression of all the three genes is reversed in the mutant embryos [103]. All the reports suggest that a normal level of DNA methylation is required for controlling differential expression of the paternal and maternal alleles of imprinted genes.

The significance of DNA methylation in carcinogenesis will be discussed in details in part 2.2.

2.1.4 Approaches to assess DNA methylation

When DNA methylation has become a very hot research area, quite a few techniques have been developed to analyze the methylation status either genome-wide or for a specific gene. Therefore methods for DNA methylation analysis can also be divided roughly into two categories: global and gene-specific methylation analysis.

For gene-specific methylation analysis, the earliest studies used methylation sensitive restriction enzymes (MSRE) to digest DNA followed by Southern or PCR detection. The MSREs are able to cleave DNA at specific methylated CpGs which have lost the methyl group. The digested products can be amplified by PCR or detected by Southern [104]. This will result in differentiating the methylated versus non-methylated DNA. The major disadvantage of this method is incomplete digestion of DNA by the MSRE, which will

lead to false-positive result. Besides the use of MSRE, bisulfite reaction based methods, such as methylation-specific PCR and bisulfite sequencing, are more frequently used now.

For global methylation analysis, the affinity enrichment using methyl-binding domain or anti-5-meC antibody, and microarray strategies are frequently used. For example, the Methylated DNA Immunoprecipitation (MeDIP) using 5-methylC antibody allows direct comparison of immunoprecipitated DNA and methylation status [105-107]. Proteins with methyl DNA binding domain (MBD) are also very useful tools to be incorporated into purification of methylated DNA [108]. Both natural and some engineered MBD proteins can be used to fractionation of methylated DNA by the CpG density [109]. These strategies can be combined with either PCR or Southern for single gene analysis, or microarray approaches for genome-wide analysis, for example, a CpG island microarray [110].

The latest principles and classification of DNA methylation analysis are listed in Table 2.1 [111].

Table 2.1 Main principles of DNA methylation analysis

Pretreatment	Analytical step			
	Locus-specific analysis	Gel-based analysis	Array-based analysis	NGS-based analysis
Enzyme digestion	<ul style="list-style-type: none"> • HpaII-PCR 	<ul style="list-style-type: none"> • Southern blot • RLGS • MS-AP-PCR • AIMS 	<ul style="list-style-type: none"> • DMH • MCAM • HELP • MethylScope • CHARM • Mmass 	<ul style="list-style-type: none"> • Methyl-seq • MCA-seq • HELP-seq • MSCC
Affinity enrichment	<ul style="list-style-type: none"> • MeDIP-PCR 		<ul style="list-style-type: none"> • MeDIP • mDIP • mCIP • MIRA 	<ul style="list-style-type: none"> • MeDIP-seq • MIRA-seq
Sodium bisulphite	<ul style="list-style-type: none"> • MethyLight • EpiTYPER • Pyrosequencing 	<ul style="list-style-type: none"> • Sanger BS • MSP • MS-SNuPE • COBRA 	<ul style="list-style-type: none"> • BiMP • GoldenGate • Infinium 	<ul style="list-style-type: none"> • RRBS • BC-seq • BSPP • WGSBS

AIMS, amplification of inter-methylated sites; BC-seq, bisulphite conversion followed by capture and sequencing; BiMP, bisulfite methylation profiling; BS, bisulfite sequencing; BSPP, bisulfite padlock probes; CHARM, comprehensive high-throughput arrays for relative methylation; COBRA, combined bisulfite restriction analysis; DMH, differential methylation hybridization; HELP, HpaII tiny fragment enrichment by ligation-mediated PCR; MCA, methylated CpG island amplification; MCAM, MCA with microarray hybridization; MeDIP, mDIP and mCIP, methylated DNA immunoprecipitation; MIRA, methylated CpG island recovery assay; Mmass, microarray-based methylation assessment of single samples; MS-AP-PCR, methylation-sensitive arbitrarily primed PCR; MSCC, methylation-sensitive cut counting; MSP, methylation-specific PCR; MS-SNuPE, methylation-sensitive single nucleotide primer extension; NGS, next-generation sequencing; RLGS, restriction landmark genome scanning; RRBS, reduced representation bisulfite sequencing; -seq, followed by sequencing; WGSBS, whole-genome shotgun bisulfite sequencing [111].

2.2 DNA hypermethylation in HBV-associated HCC

2.2.1 DNA hypermethylation in HCC

Aberrant DNA methylation is an important epigenetic alteration in HCC development [112]. Studies have established the methylation profiles of many TSGs in aging liver, chronic hepatitis and HCC. These indicate that many of the TSGs are usually hypermethylated in carcinogenesis including HCC [54-56]. For instance, significant link has been reported between the HCC development and the silence by DNA hypermethylation of several TSGs, such as IGFBP-3, RASSF1A [22], p16^{INK4a} [57], p15 [58], E-cadherin [59, 60], GSTP1 [61] and DLEC1 [62]. Among these, RASSF1A is hypermethylated in the early phase of HCC while p16^{INK4a} is hypermethylated in the late phase of HCC [47, 63]. These data suggest that DNA methylation which occurs in the early stage of cancer development including HCC may also be an area of interest for biomarker identification.

2.2.2 DNA methylation in HBV-associated HCC

The involvement of HBV in HCC development has been indicated by numerous clinical and laboratory studies. However, aberrant epigenetic modifications of target genes in HBV-associated HCC have only been reported in the past several years.

An epigenetic model of the HBV-associated HCC development has recently been proposed, in which genome wide hypomethylation increases chromosome instability while regional hypermethylation decreases tumor suppressor gene expression thus increasing the risk of HCC development [22]. A significant link has been suggested between HCC development and the silencing by DNA hypermethylation of several TSGs. Interestingly, most of the abnormality of DNA methylation status of these genes, including p15, p16 [20, 70-72], E-cadherin [21, 59], RASSF1A, IGFBP-3 [22], and GSTP1 [2, 61], has been further associated with HBV infection. Target genes regulated by HBV via DNA methylation and possible mechanisms involved have been summarized in Fig.1.4, modified from a proposed model [20]. The role for individual HBV proteins in regulating DNA methylation, in particular HBx, has recently been suggested. Some of the above mentioned TSGs, including E-cadherin and p16^{INK4a}.

Although most of the published reports have indicated that HBV/HBx increases DNA methylation of various target genes, the methylation status of CpG islands of a number of genes could be decreased by HBV infection. SOCS-1 (suppressor of cytokine signaling-1) hypermethylation is found to be positively associated with HCV infection but inversely associated with HBV infection in tissues from HCC patients [47]. In addition, the hypomethylation of serum LINE-1 (long interspersed nuclear element-1) correlates significantly with the HBV infection [113]. Recent proteomic analysis has suggested that aldehyde dehydrogenase 1 (ALDH1) is hypomethylated by HBx [114]. Besides DNMT1, *de novo* DNA methyltransferase DNMT3A has also been implicated in the HBx mediated epigenetic modification. Conflicting data have been reported on a putative tumor

suppressor IGFBP-3, which mediates growth suppression signals in the IGF-1/IGF-1R pathway. IGFBP-3 has been found to be repressed by HBx through *de novo* DNA methylation by DNMT3A1 and DNMT3A2 [22]. On the other hand, the transcription of IGFBP3 can be triggered by HBx via deprivation of DNMT3A from their promoters [23]. Two other genes, interleukin-4 receptor and metallothionein-1F, are also found to be silenced by HBx via recruiting DNMT3A [23].

2.2.3 Proteomic analysis of HBV-associated HCC

Most epigenetic studies on HBV-associated HCC have been carried out on the basis of evaluating candidate genes individually [115]. Thus a comprehensive understanding on the underlying mechanisms of HCC development remains to be established [116]. Comprehensive understanding of the disease pathogenesis can be analyzed by various -omics approaches, including transcriptomics and proteomics. For transcriptomics, microarray analysis is often used to detect alterations in mRNA expression of genes. Recently, the proteomics analysis has been popular and people believe that proteomics may provide more definite clues because critical functional and structural activities of most organisms reside in proteins [117].

The 2-D electrophoresis (2-DE) coupled with mass spectrometry (MS) analysis has been the most widely used proteomic approach to profile the global protein expression alterations in HBV-associated HCC clinical samples [117-119]. Later developments have

evolved from gel-based 2-D difference in-gel electrophoresis (DIGE) [117, 120] and isotopo-coded affinity tags (ICAT) [121], to the recent gel-less techniques including the matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-ToF/MS) [122] and iTRAQ (Isobaric tags for relative and absolute quantification) -coupled 2-D LC-MS/MS (2-dimensional High-performance liquid chromatography with tandem mass spectrometry) analysis [2]. The gel-less techniques have shown significant improvements in particular in the accuracy of quantification, as well as the high-throughput analysis of low-abundance samples in some clinical proteomics [123]. The conventional proteomics analysis on HBV-associated HCC has mainly been based on comparing protein profile in cancerous and noncancerous samples [124], mostly on tissues [60, 114, 125]. Sera from HCC patients have also been subjected to similar comparative analysis [126-128]. Results from such a comparative analysis have revealed up- or down-regulated proteins in cancer or non-cancer samples, and their possible correlation with HBV infection.

Microarray analysis has been used to compare genes down-regulated in tumor-conditioned versus quiescent endothelial cells, and re-expressed by 5-Aza-dC (DNMT inhibitor) and TSA (trichostatin, histone deacetylase inhibitor) [85]. As a complement to microarray analysis, proteomics can be used to analyze the epigenetic regulated target proteins by HBV in HBV-associated HCC.

2.3 Clinical implications of DNA hypermethylation in HCC

2.3.1 Methods for Diagnosis

Alpha-fetoprotein (AFP) has been widely used for the screening of HCC. However, accuracy of AFP-based screening has not been satisfactory [34, 129]. This has been shown in false negative cases which are in the early stage of HCC [117]. Thus it is important to identify new biomarkers for HCC to achieve credible early diagnosis which allows early effective treatment. Apart from many proteomic studies done to identify novel protein candidates as biomarker of HCC, DNA methylation as an important feature of alteration which could happen in the very early stage of cancer development, has been paid much attention and could also be used as biomarker for HCC.

Several DNA hypermethylation detecting methods have been developed during recent years, genome-wide and quantitative, high-resolution methods for methylation analysis are used both in the lab and clinic [130], among which Methylation-Specific PCR (MSP) is considered as the relatively inexpensive and highly sensitive one for diagnostic application [131]. Briefly, MSP includes sodium bisulfite treatment of DNA, and this treatment can convert unmethylated cytosine to uracil, while methylated cytosine remain cytosine, as shown in Fig. 2.3. Before the modified DNA amplified by PCR, specific primers to distinguish between methylated and unmethylated DNA should be designed. Using such primers and template DNA which have been modified by the bisulfite treatment, the PCR can be performed [131]. MSP is a sensitive and specific technique and

therefore has been widely used to get methylation patterns in many reported studies.

Figure 2.3 Principle of Methylation-Specific PCR. (A) After treatment of sodium bisulfite, unmethylated cytosine converts to uracil while methylated cytosine remains cytosine. (B) Results are indicated by PCR fragment detected with unmethylated (U) or methylated (M) primer sets.

The high sensitivity of MSP allows the sample materials to be patient DNA from blood, sputum or urine. These samples are non-invasive to get, so it is suitable for early screening in populations. It is reported that the hypermethylation of two TSGs, RASSF1A and p16^{INK4a}, has been used as markers to help diagnosis of cancer [132].

2.3.2 Treatment

The fact that methylation-induced gene silencing can be rescued by demethylation and reactivated makes DNMT inhibitors promising therapeutic agent against cancer

development. The best known DNMT inhibitors are 5-azacytidine (5-Aza-C) and 5-aza-2'-deoxycytidine (5-Aza-dC) (Fig. 2.4A), which are both analogues of cytosine.

The proposed inhibitory mechanism is by covalent enzyme trapping [90]. The drugs are first converted into the corresponding nucleotides and then incorporated into DNA (5-Aza-dC), while 5-Aza-C is mostly incorporated into RNA and to a lesser extent into DNA. The drug-incorporated DNA can covalently bind the DNMTs but not release. Therefore these DNA methyltransferases are no longer available or even degraded when the methylation pattern is being copied to the newly synthesized DNA strands during DNA replication. Accordingly, the methylation signature is successively lost over the following cycles of DNA replication [38]. Another explanation is 5-Aza-C/dC blocking (Fig. 2.4B) [55]. When the compound is integrated into DNA, replacing the natural base cytidine, it acts as a direct and irreversible inhibitor of the DNMTs, since it contains a nitrogen in place of carbon at the 5 position of the cytidine ring [55].

Figure 2.4 Structures of 5-azacytidine and 5-aza-2 deoxycytidine and their effects of inhibiting methylation. (A) Structures of 5-azacytidine and 5-aza-2 deoxycytidine. 5-azacytidine has a –OH group while 5-aza-2 deoxycytidine has a –H instead. [38] (B) The function of blocking DNMTs of 5-azacytidine. DNMTs use S-adenosyl-methionine as the donor molecule for the methyl group. This reaction can be blocked by the drug 5-azacytidine [55].

If DNMT inhibitors are used to reactivate the expression of those epigenetically silenced genes, significant clinical benefits may be expected. Clinical trials using DNMT inhibitors have shown beneficial effects in drug-treated patients [133, 134], despite side-effects [135]. Alternative novel DNMT inhibitors are being explored for less toxic side-effect and better specificity.

2.4 Conclusions

Recent progress in the field of epigenetics has provided valuable insights into the pathogenesis of HCC, as well as the mechanism of HBV involvement. Detailed mechanisms of the HBV-mediated DNA methylation in HCC development need to be further explored. In addition to the reported studies based on individual genes or proteins, comprehensive profile of target protein need to be established to provide a better understanding of epigenetic alterations in HBV-associated HCC which will help its epigenetic therapies.

Chapter 3 Materials and Methods

The following section contains an overall description of the materials and methodologies used in this project, including proteomic techniques and analysis, general methods in molecular biology, and some specific techniques for methylation studies.

For proteomic studies, high-throughput proteomic level exploration equipped with High-performance Liquid Chromatography with tandem Mass Spectrometry (LC-MS/MS) analysis was used to establish the protein expression profile in cells after transfection or treatments. Molecular biology techniques include sub-cloning, cell culture, DNA/RNA handling, RT-PCR, Real-time RT-PCR, Western blotting, Microparticle Enzyme Immunoassay (MEIA), Luciferase Reporter Assay, reactive oxygen species detection (ROS assay) and Fluorescence-activated cell sorting (FACS). Methylation-specific PCR (MSP), Bisulfite Genome Sequencing (BGS) and chromatin immunoprecipitation (ChIP) were also used to analyze the epigenetic status of interested genes under certain conditions. All the detailed procedures of each method and materials as well as equipments used are listed as follows:

3.1 Research Methodology

3.1.1 Cloning

3.1.1.1 HBV DNA from clinical samples

The HBV whole genome and coding sequence of HBV X protein (HBx) with various genotypes stored in our lab are extracted from serum samples of HBV-infected patients provided by National University Hospital (NUH), Singapore. The whole genomes of genotype A-C were cloned into mammalian expression vector pcDNA3.1 (Invitrogen) by our senior colleagues Dr. Pan Hong (genotype A) and Dr. Tan Tuan Lin (genotype B and C). HBx constructs were designed by me and experiments were performed by Ms. Feng Huixing. Specifically, HBx coding sequences with genotypes A-C were amplified by PCR from the above mentioned HBV genome constructions, while HBx genotype D was obtained directly from clinical serum samples by PCR amplification and inserted into Hind III/Pst I (New England Biolabs) sites of another vector named pXJ40 with higher expression efficiency and HA tags, which was a kind gift from Dr. Cheng Gee Koh (SBS, NTU, Singapore) [136, 137].

The primers used for amplifying HBx coding sequences as listed in Table 1 were designed with the help of an online free software Primer 3 (v 0.4.0) (<http://frodo.wi.mit.edu/primer3/>). The whole HBx coding sequence excluding the ATG

(aiming the fusion with HA tag at the 5' end) was cloned into pXJ40 vector at Hind III/Pst I sites. The three extra bases in each primer at 5' end were included as protecting bases to ensure the normal digestion of the restriction endonucleases (Hind III and Pst I).

Table 3.1 Primers used for plasmid construction

Primer Name	Sequence
HBx A forward	5'-CCCAAGCTTGCTGCTAGGCTGTACT-3' (Hind III)
HBx B forward	5'-CCCAAGCTTGCTGCTAGGCTGTGCT-3' (Hind III)
HBx C forward	5'-CCCAAGCTTGCTGCTAGGGTGTGCT-3' (Hind III)
HBx D forward	5'-CCCAAGCTTGCTGCTAGGCTGTGCT-3' (Hind III)
HBx A/B/C/D reverse	5'-AACTGCAGTTAGGCAGAGGTGAAA-3' (Pst I)

3.1.1.2 Vectors used for cloning

Three vectors were used for cloning in this project. They are mammalian expression vectors pcDNA3.1 (Fig. 3.1, Invitrogen) and pXJ40 (Fig. 3.2, gift from Dr. Cheng Gee Koh; SBS, NTU, Singapore) for expression proteins in cell lines; and pGEM-T Easy Vector (Fig. 3.3, A1360, Promega) for rapid ligation of PCR products. The maps of the vectors are listed as below:

Figure 3.1 Feature map of mammalian expression vector pcDNA3.1. Gene of interest could be inserted into the multiple cloning sites after T7 promoter. T7 promoter/priming site allows for in vitro transcription and help sequencing confirmation of the inset. The expression of insert gene is driven by CMV promoter. BGH benefits efficient transcription termination and polyadenylation of mRNA. Ampicillin resistance gene is included for selection of clones positively containing insert fragment. Neomycin resistance gene is for selection of stable cell lines using neomycin (Geneticin). And SV40 promoter is for expression of neomycin resistance gene. pUC origin is for high-copy number replication and growth in *E. coli*. (Commercially available from Invitrogen).

Figure 3.2 Feature map of mammalian expression vector pXJ40. Gene of interest could be inserted into the multiple cloning sites after T7 promoter. The expression of insert gene is driven by HCMV promoter. Ampicillin resistance gene is included for selection of clones positively containing insert fragment. (Gift from Dr. Cheng Gee Koh, SBS, NTU, Singapore).

Figure 3.3 Feature map of pGEM-T Easy vector. Inserted fragments within the sequence of the enzyme β -galactosidase can cause inactivation of the enzyme β -galactosidase, which allows identification of recombinants by blue/white screening on indicator plates. Ampicillin resistance gene is included for selection of clones. (Commercially available from Promega).

3.1.1.3 PCR purification

In cases where the PCR products were not very specific, the required band was separated from others by gel purification procedures (see part *3.1.1.5 DNA purification by agarose gel extraction*). For very specific PCR reactions, the desired DNA fragment was directly purified with PCR purification kit (QIAGEN). Briefly, five volumes of Buffer PB were added to one volume of the PCR sample. The mixture was then applied to a QIAquick spin column and centrifuged for 30-60 sec at 10000 rpm. The flow-through was discarded and 0.75 ml Buffer PE was added to the column followed by centrifugation for 30-60 sec. The flow-through was discarded again. Column was put back into collection tube and centrifuged for 1 min at 13200 rpm to remove any excess ethanol. After that, column was put in a clean 1.5 ml microcentrifuge tube and 30-50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or H₂O was added to the center of the membrane and centrifuged for 1 min to elute DNA.

3.1.1.4 Endonucleases Digestion and CIP treatment

The purified PCR fragments and empty vectors were digested respectively by certain endonucleases as designed. The digestion was performed according to the products' instructions. Generally, the purified PCR fragment or 3-5 µg of vector plasmid was digested in a 25-50 µl volume, with 1×NEBuffer, 5-10 unit of endonuclease, and 1×BSA (if necessary) , at 37 °C for 1-2 hr.

Vector DNA was further dephosphorylated with calf intestinal alkaline phosphatase (CIP) by adding 1-2 μ l of the CIP into digestion reaction, and incubated at 37 °C for another 30-60 min. This treatment could release 5' phosphate group of DNA fragment and significantly reduce the rate of self-ligation of vectors in the subsequent ligation reaction.

3.1.1.5 DNA purification by agarose gel extraction

PCR fragments, and vectors after digestion and CIP treatment, were then run in agarose gel and purified by QIAquick Gel Extraction kit (QIAGEN). DNA band slice was excised from the gel with a scalpel and weighed in a 1.5 ml microcentrifuge tube. To one volume of gel (100 mg ~ 100 μ l), three volumes of Buffer QG were added. The mixture was then incubated at 50 °C for 10 min, during which mixture was mixed by vortexing every 2-3 min. After gel slice dissolved completely, one gel volume of isopropanol was added and mixed. Then the mixture was applied to QIAquick column and centrifuged for 1min to bind DNA. Flow-through was discarded and column was washed by 0.5 ml of Buffer QG and 0.75 ml of Buffer PE. After washing, column was centrifuged for an additional 1 min at 13,000 rpm and placed into a clean 1.5 ml microcentrifuge tube. Finally, 50 μ l Buffer EB or H₂O was added to the column, centrifuged for 1 min. The flow-through was the purified DNA.

3.1.1.6 Ligation

Purified PCR fragment and linear vector were ligated with T4 DNA ligase (M0202S, NEB). For each ligation, a 10 µl reaction was prepared by adding 1 µl of T4 DNA ligase (400 cohesive end units/µl, 1 µl of 10×T4 DNA ligase buffer, 1 µl of vector (50-100 ng), and 7 µl of insert (PCR fragment). The mixture was mixed and incubated at room temperature for 1-2 hr, or at 4 °C or 16 °C for overnight.

For the ligation with pGEM-T Easy vector, pGEM-T Easy Vector System I (A1360, Promega) was used. The reaction was 5 µl of 2×rapid ligation buffer, 0.5 µl of pGEM-T Easy vector (50 ng), 0.5 µl of T4 DNA ligase, and 4 µl of PCR fragment. The mixture was mixed and incubated at room temperature for 1 hr, or at 4 °C for overnight.

3.1.1.7 Preparation of competent E. coli cells

The CaCl₂ method was used to prepare competent cells, as previously described in Molecular Cloning: A Laboratory Manual [138].

E. coli DH5α stock strain was streaked onto a fresh LB agar plate (without antibiotics) and incubated at 37 °C overnight (the preparation of agar plate will be described in part 3.2). After that, a single clone was picked out from the agar plate and inoculated into 5 ml of LB medium without any antibiotics and incubated at 37 °C overnight with shaking at 220-250 rpm. The next day, 1 ml of the amplified bacteria were re-inoculated into fresh 100 ml LB medium at a ratio of 1:100; and allowed to grow to a concentration of OD₆₀₀

between 0.3-0.4 (took about 2-3 hr), to make sure that the culture was in exponential phase. When it was ready, the 100 ml culture was aliquoted into 50 ml pre-chilled centrifuge tubes and incubated on ice for 10 min, followed by centrifugation at 4 °C, 5000 rpm for 5 min. Cells were kept cold for the rest of the procedures: transporting tubes and resuspending cell pellets on ice. Cell pellets were then well resuspended in 30 ml of 0.1 M ice-cold MgCl₂ by gently flicking, followed by another centrifugation at 4 °C, 5000 rpm for 5 min. The supernatant was discarded. The cell pellets were resuspended again with 20 ml 0.1 M ice-cold CaCl₂ and incubated on ice for 30 min. After incubation, the solution was centrifuged once more at 4 °C, 5000 rpm for 5 min. And supernatant was discarded and cell pellets were resuspended again in 1.5 ml (cells in each 50 ml resuspended in 1.5 ml) of 0.1 M ice-cold CaCl₂/15% glycerol by gently pipetting. The resulting cell solution contained the prepared competent cells, and could be used immediately or aliquoted into autoclaved fresh tubes and stored at -80 °C.

3.1.1.8 Transformation

Competent cell *E. coli* DH5 α was used for transformation after the ligation. After cells were thawed on ice, the 10 μ l ligation reaction was added into the competent cells by pipetting gently, and incubated on ice for 60 min. Meanwhile, a 42 °C water bath was set for heat shock. After incubation, the tube containing cells and ligation mixture was put in 42 °C for 90 sec. The tube was then taken out from the water bath and put on ice for another 90 sec. 800 μ l of LB medium without antibiotics was added into the mixture and

incubated at 37 °C for 1 hr with rotation of 250 rpm. Cells were then pelleted by centrifugation at 400 rpm for 1 min at room temperature. Most of the supernatant was discarded but leaving around 100 µl. Cell pellet was resuspended in the 100 µl of supernatant (LB medium) and spread on LB agar plate with suitable selective antibiotics. The plate was then incubated at 37 °C overnight.

3.1.1.9 Mini-preparation of plasmids

Clones on the plate after overnight incubation were selected and each of the clone was inoculated into 5 ml LB medium for a overnight incubation at 37 °C. Mini-preparation of the plasmids was carried out with QIAprep plasmid miniprep kit (QIAGEN). Briefly, cells from overnight culture were collected by centrifugation at 10000 rpm for 1 min. Cell pellet was resuspended by 250 µl of Buffer P1 (with RNase A) and mixed by vortexing or pipetting. 250 µl of Buffer P2 was added and mixed by inverting the tube for several times. 350 µl Buffer N3 was added and immediately mixed thoroughly by inverting to get a homogeneous colorless suspension. The mixture was then centrifuged for 10 min at 13000 rpm and the supernatant was applied to the QIAprep spin column by pipetting. The column was centrifuged for 30-60 sec and the flow through was discarded. The column was then washed first by 0.5 ml of Buffer PB, then 0.75 ml of Buffer PE by centrifuging for 30-60 sec. The flow through was discarded and centrifuged for 1 min to remove excess reagent. Finally, 50 µl of Buffer EB or H₂O was added to the column, followed by a centrifugation for 1 min. The flow through was the plasmid DNA mini-prepared and

could be checked by restriction endonuclease digestion and DNA electrophoresis or sequencing.

3.1.1.10 DNA sequencing and sequence analysis

Plasmids containing interested genes or promoters were confirmed by sequencing, service provided by National Neuroscience Institute research lab, Singapore. Sequences were analyzed and aligned with the software Vector NTI Suite (v. 6.0, InfoMax Inc.), according to the guidelines of the software manuals. Briefly, all the interested sequences from NCBI Genbank database (<http://www.ncbi.nlm.nih.gov/Genbank/>) can be imported into the software and different site-restricted enzymes cutting sites and all the sequence elements (including 3'-UTR, 5'-UTR, coding regions, poly A tails and so on) could be viewed with the Analyze function of the software during the design of a cloning. Once a cloning has been done and sequenced, the sequencing results can also be imported into the software. By using the Align function of the software, we can align selected sequences with the original one, so as to check the correctness of the cloning or to check if the interested CpG sites were methylated or not.

3.1.1.11 Midi-preparation of plasmids

For transfection of mammalian cells, plasmids prepared by midi-preparation were used. The midi-preparation was performed with QIAGEN plasmid Midi kit (QIAGEN)

according to manufacturer's instructions.

Usually 25-50 ml of cultures was prepared for a midi-prep. The bacterial cells were collected by centrifugation at 6000 g for 15 min, 4 °C and resuspended thoroughly in 4 ml of Buffer P1. 4 ml of Buffer P2 was added and sample was mixed by inverting the sealed tube 4-6 times and then incubated at room temperature for 5 min, followed by adding 4 ml of chilled Buffer P3 and mixed by inverting again and incubated for 15 min on ice. The mixture was centrifuged at 20000 g (4 °C, 30 min). During the centrifugation, a QIAGEN-tip 100 column was equilibrated by adding 4 ml of Buffer QBT and allowed to empty by gravity flow. After centrifugation, the supernatant containing plasmid DNA was moved to the column which had been equilibrated, and allowed to enter the resin by gravity flow. The column was then washed with 10 ml Buffer QC for twice, and after that, DNA was eluted from the column by 5 ml Buffer QF. The DNA was further precipitated by applying 3.5 ml (0.7 volumes) isopropanol, mixed and centrifuged immediately at 15000 g for 30 min at 4 °C. The supernatant was carefully decanted and DNA pellet was washed with 2 ml of room-temperature 70% ethanol, centrifuged at 15000 g for 10 min. Supernatant was removed carefully and the pellet was air-dried for 5-10 min. DNA pellet was then re-dissolved in buffer TE (volume according to the amount of DNA) and the resulting solution was the midi-prepared plasmid.

3.1.1.12 Quantification of DNA

Quantity and quality of DNA were determined by both UV spectrophotometer readings at 260 nm/280 nm and agarose gel analysis. The purity of the DNA extract was evaluated by spectrophotometer determination of the A_{260}/A_{280} ratio (1.7-1.8), while the concentration was calculated according to the A_{260} according to the formula (for double strand DNA):

$$\text{DNA concentration } (\mu\text{g/ml}) = A_{260} \times 50 \times \text{dilution factor.}$$

3.1.2 Cell work

3.1.2.1 Cell freezing, storage and recovery

Mammalian cell lines were purchased from American Tissue Culture Collection (ATCC) and kept in liquid nitrogen for long term storage. To recover the cells for culture, the tube containing frozen cells was moved out of liquid nitrogen and put into 37 °C water bath immediately to allow the cells to be thawed in less than 2 min. The following steps were carried out in a biological safety cabinet to prevent any contamination. After thawing, the cell solution was transferred into a fresh centrifuge tube and another 5 ml of culture medium (e.g., minimal essential medium (MEM) supplemented with 10% fetal

bovine serum (FBS)) was added, followed by centrifugation of 800 rpm for 3-5 min. The supernatant was discarded and cell pellet was resuspended in 10 ml of fresh culture medium and transferred to a new 10 cm culture dish.

For cell storage, cells were frozen and kept in liquid nitrogen. Briefly, cells were trypsinized and resuspended in 10 ml of culture medium, then centrifuged at 800 rpm for 3-5 min. The cell freezing medium was prepared freshly: 50% MEM, 40% FBS, 10% DMSO. After centrifugation, the supernatant was discarded and cell pellet was resuspended with suitable amount of freezing medium and aliquoted into cell freezing tube. After that, the freezing tube was put in 4 °C for 30-60 min, and -20 °C for 1-2 hr, then -80 °C overnight. The next day, the tube could be moved into liquid nitrogen for long term storage.

3.1.2.2 Cell culture and cell passage

Cell lines used in this project except HepG2.2.15 were kept with minimal essential medium (MEM, Gibco, Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen); and HepG2.2.15 was maintained with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 1×Non-essential amino acid (Gibco, Invitrogen), and 150 µg/ml of G418 (Geneticin, Gibco, Invitrogen), in an incubator containing 5% CO₂ at 37 °C.

For cell passage, the medium was removed and cells were washed by $1\times$ Phosphate buffered saline (PBS) for one time, then 0.1% Trypsin with EDTA (Ethylenediaminetetraacetic Acid) was added to dish with attached cells and cells were incubated at $37\text{ }^{\circ}\text{C}$ for 2-5 min. Cells were resuspended with 10 ml of medium and centrifuged at 800 rpm for 3-5 min. The supernatant was discarded and cells were resuspended again with 10 ml of fresh medium and aliquoted into more dishes for culture.

3.1.2.3 Cell number determination

To determine the cell number in a dish, cells were trypsinized and resuspended in certain amount of medium. The medium solution with cells was mixed well by pipetting, and 50 μl of the cell suspension was diluted by adding 450 μl of medium. 10 μl of the diluted cell suspension was loaded into the hemocytometer chamber (between the cover slip and counting area). Cell number in every 4×4 square was counted under microscope, and an average number was calculated according to all the counted numbers. The cell concentration in the original cell suspension was calculated according to the following formula:

$$\text{Cell number (/ml)} = \text{Average number of cells in big square} \times 10^4 \times \text{Dilution factor.}$$

3.1.2.4 5-Aza-2'-Deoxycytidine (5-Aza-dC) and Trichostatin A (TSA) treatment of the cells

Cells were seeded in low density 12-18 hr before treatment. Cells were treated from a 10 mM Dimethyl Sulphoxide (DMSO) (Sigma, USA) dissolved stock solution of 5-Aza-dC (A3656, Sigma) at a final concentration of 5-10 μ M, or were mock-treated by adding into the medium the same volume of DMSO alone. Treatment was conducted for 96 hr, with the drug and culture medium replaced every 24 hr, as described previously [61, 139]. For the TSA treatment, the cells were treated from a DMSO-dissolved stock solution of 5 mM TSA (T8552, Sigma-Aldrich) at a final concentration of 500 ng/ml [140, 141], or were mock-treated by adding into the medium the same volume of DMSO only. Treatment was conducted for 24-48 hr, with the drug and culture medium replaced every 24 hr.

3.1.2.5 Cell transfection

For the transfection, different methods were used for different cell lines, so as to achieve better transfection efficiency.

For HepG2 and Huh7 cells, electro-transfection from Amaxa system was used following the protocol provided by the Cell Line Nucleofector Kit V (Amaxa Inc, USA); and about 40% efficiency could be achieved for HepG2, while 80-85% for Huh7. Briefly, after seeding for 14-18 hr, $5-6 \times 10^6$ cells were trypsinized and collected by centrifugation

at 800 rpm for 3-5 min. The cell pellet was resuspended in 100 μ l of Nucleofector Solution V. 2-4 μ g of highly purified plasmid was added into the cell suspension, mixed by gently pipetting, and transferred to the Amaxa certified cuvette provided with the kit. Cell-type specific program was chosen and the cuvette was inserted into the Nucleofector started the program by pressing the “X” button. After that, the sample was immediately removed with an Amaxa certified pipette from the cuvette with 500-800 μ l pre-warmed medium, and transferred to 10 cm culture dish for culture at 37 °C. After 4-6 hr, when the cells had attached to the surface of dish, supernatant was discarded and then fresh medium was added. Expression after transfection could be allowed to last 24-48 hr before cells were collected for down-stream experiments.

For 293T cells, the Effectene Transfection Reagent (QIAGEN) was used following the protocol provided with the kit, and the transfection efficiency could reach 90%. Briefly, $2-8 \times 10^5$ cells were seeded per 60 mm dish the day before transfection and make the confluence of cell 50-60% on the day of transfection. 1 μ g plasmid DNA was diluted with Buffer EC (DNA condensation buffer) to a total volume of 150 μ l; 8 μ l Enhancer was added and mixed by vortexing for 1 sec. The DNA-enhancer mixture was incubated for 2-5 min and then 25 μ l Effectene Transfection Reagent was applied to the mixture by pipetting and incubated for another 5-10 min to allow the transfection-complex formation. During this, cells were washed by 4 ml of PBS and 4 ml fresh medium was added to cells. 1 ml medium was added to transfection complexes and after mixing, the complexes were added to the cells drop-wise with swirling the dish. Cells were incubated at their normal growth conditions for 24-48 hr for expression of the transfected gene.

3.1.3 IMx analysis of HBs Ag in culture medium

The secretion of Hepatitis B Surface Antigen (HBs Ag) in the culture medium of HepG2.2.15 was detected by using IMx immunoassay system (Abbott, USA) and IMx HBs Ag Kit (Abbott, USA), a qualitative Microparticle Enzyme Immunoassay (MEIA) for the detection of HBs Ag. Culture medium of HepG2 and HepG2.2.15 was collected and centrifuged to remove insoluble cell debris. When HBs Ag was present, it bound to the Anti-HBs Coated Microparticles becoming a complex. Then the biotinylated anti-HBs solution was dispensed to the reaction mixture becoming an antibody-antigen-antibody complex. A portion of the reaction mixture bound to the microparticles would then be transferred to the glass fiber matrix, to which the microparticles bound irreversibly. Anti-Biotin-Alkaline Phosphatase (AP) conjugate was dispensed onto the matrix and bound to the antibody-antigen-antibody complex. The matrix was further washed so the unbound materials were removed. Finally, the fluorescent product was measured by the MEIA optical assembly after the substrate of AP (4-Methylumbelliferyl Phosphate) was added to the matrix.

3.1.4 Western Blot analysis

Cell extracts were prepared in a home-made TNT lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton-100, and a protease inhibitor cocktail), followed by the

protein concentration quantification with Bradford Dye Reagent (#500-0205, Bio-Rad). After that, equal amounts (30 µg) of cell lysates were resolved by 7.5%-12% SDS-PAGE. The resolved proteins were semi-dry electro-transferred to Polyvinylidene Fluoride (PVDF) membranes (Bio-Rad), which were then blocked in a 50 ml tube by 5 ml of 1×PBST (1×PBST contains 3.2 mM Na₂HPO₄, 0.5 mM KH₂PO₄, 1.3 mM KCl, 135 mM NaCl, 0.05% Tween 20, pH 7.4) rolling on a roll and tilt mixer at room temperature for 1-2 hr. After blocking, the membrane was probed with primary antibody in dilution of 1:200-1:10000 (dilution range depending on different primary antibody and the abundance of interested proteins) on a roll and tilt mixer at 4 °C overnight. The next day, membrane was incubated with a proper secondary antibody at room temperature for 1 hr, either a horseradish peroxidase (HRP)-conjugated anti-goat IgG antibody (#31402, PIERCE), or an anti-rabbit IgG antibody (#31460, PIERCE), or an anti-mouse IgG antibody (#31430, PIERCE) in 1: 5000 dilution. After secondary antibody, membrane was incubated with substrates of HRP, Supersignal West solutions (#1856136, PIERCE). Two Supersignal West solutions was mixed at ratio of 1:1 in a 1.5-2 ml microcentrifuge tube, and added on a piece of plastic cling wrap drop-wise, on which the membrane was laid down without creating air bubble between the membrane and substrate solution. The membrane was incubated with the substrate in dark for 3-5 min, and wrapped with a fresh piece of cling wrap. The following steps were then carried out in dark room. Membrane was exposed to a piece of X-ray film (Thermo Scientific Pierce) for a few seconds, and the X-ray film was then dipped into developer buffer and fixer buffer (Kodak) sequentially until clear bands could be seen on the film. The time of exposure, as well as the time in developer and fixer buffers was adjusted for different cases to achieve the best

results. For quantification analysis of Western results, a software Quantity One 4.6.2 (Bio-Rad) coupled with a scanner was used according to the manual provided.

3.1.5 RNA extraction and quantification

RNA of the cells was isolated using RNeasy Mini kit (QIAGEN) following the product manual. Briefly, buffer RTL was added to cell pellet, followed by addition of 1 volume 70% ethanol. The mixture was transferred to RNeasy mini column and centrifuged at a bench top centrifuger (Sigma). The flow-through was discarded. After that, buffer RW1 and buffer RPE were used to wash the column respectively. RNase-free water was added directly onto the membrane of the column and RNA was eluted by centrifugation.

The quality of RNA was examined by measuring in a spectrophotometer the Ratio of absorbance at 260 nm to 280 nm (1.8-2.0), while the concentration was calculated according to the A_{260} according to the formula (for single strand RNA):

$$\text{RNA concentration } (\mu\text{g/ml}) = A_{260} \times 40 \times \text{dilution factor.}$$

3.1.6 RT-PCR & Real-time RT-PCR

RT-PCR was carried out utilizing QIAGEN OneStep RT-PCR kit (QIAGEN), following the manual provided. Template RNA, primer solutions, dNTP mix, 5 × QIAGEN OneStep RT-PCR Buffer and RNase-free water were all thawed on ice. A master mix was made using the above reagents accordingly, and dispensed into PCR tubes. Template RNA (no more than 2 µg/reaction) was added to the individual PCR tubes. The thermal cycler conditions were: 50 °C 30 min for reverse transcription; 95 °C 15 min for initial PCR activation; then 30-40 cycles of 94 °C 30 sec, 50-68 °C 30 sec, 72 °C 30-60 sec; and a final extension of 72 °C 10 min. After amplification, samples could be loaded into a DNA gel and run immediately for viewing the results, or stored overnight at 4 °C, or at -20 °C for longer-term storage.

Real-time RT-PCR was carried out with iScript One-step RT-PCR kit (Bio-Rad) according to manufacturer's instructions, to accurately quantify the original amount of target mRNAs in different samples. The real-time RT-PCR was performed with the IQ5 multicolor Real-time PCR detection system (Bio-Rad). And the PCR program is: 50 °C 10 min for cDNA synthesis; 95 °C 5 min for iScript Reverse Transcriptase inactivation; then 45 cycles of PCR and detection as the following: 95 °C 30 sec, 55 °C 30 sec, 72 °C 30 sec; and a melt curve analysis of 95 °C 1 min, 55 °C 1 min, 55 °C 10 sec with 80 cycles increasing each by 0.5 °C each cycle.

Microsoft Excel formatted data which could be automatically provided by IQ5 optical system (v2.0, Bio-Rad), include amplification analysis, melt curve analysis and number of the threshold cycle. The following formula was used to calculate fold changes: Sample $\Delta Ct = Ct_{\text{sample}} - Ct_{\beta\text{-actin}}$; $\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{control}}$; the fold of sample VS control = $2^{\Delta\Delta Ct}$.

Primers used in both RT-PCR or real-time RT-PCR are listed in Table 3.2.

Table 3.2 Primers used for either real-time RT-PCR or RT-PCR

Primer Name	Sequence	Product size
HBs Ag forward	5'-TCACCATATTCTTGGAACAA-3'	1174bp
HBs Ag reverse	5'-GTTTTGTTAGGGTTTAAATG-3'	
HBc Ag forward	5'-ATCTCCTAGACACCGCCTCA-3'	132bp
HBc Ag reverse	5'-TTCCAAATTATTACCCACCC-3'	
β -actin forward	5'-CTTAGTTGCGTTACACCCTTTC-3'	151bp
β -actin reverse	5'-ACCTTCACCGTTCCAGTTTT-3'	
GSTP1 forward	5'- ATGACTATGTGAAGGCACTG-3'	256bp [142]
GSTP1 reverse	5'- AGGTTACGTACTCAGGGGA-3'	
DLEC1 forward	5'-TTCCTCCCTCGCCTACTC-3'	310bp [47]
DLEC1 reverse	5'-AAACTCATCCAGCCGCTG-3'	
CPS1 forward	5'- ATCTTCTCAGCTGTCTTGGA -3'	140bp
CPS1 reverse	5'- GACCCACTCAAAACATAGGA -3'	
His H4 forward	5'- GGACAATATCCAAGGCATTA -3'	123bp
His H4 reverse	5'- TTCTCCAGGAAGACTTTGAG -3'	
S100A6 forward	5'- GAAGGAGCTGATCCAGAAG -3'	161bp
S100A6 reverse	5'- GGGCTTCATTGTAGATCAAA -3'	
FLNB forward	5'- GCCTTAGTTCCTATGGTGTG -3'	155bp
FLNB reverse	5'- AGCATACGTGCCATCTTTAT -3'	
TKT forward	5'- GTCTTTTACCCAAGTGATGG -3'	132bp

TKT reverse	5'- CTGGAAGTCCTCATTGTTGT -3'	189bp
ANXA2 forward	5'- GACACCTGCTCAGTATGACG -3'	
ANXA2 reverse	5'- AAGTCACCAGATGTGTCCGA -3'	

3.1.7 Genomic DNA extraction and quantification

Genomic DNA of cells was extracted with PureLink Genomic DNA Kit (Invitrogen), according to the user manual provided with the kit. Genomic DNA was isolated by digestion of proteins and RNA with Proteinase K and RNase A in the Lysis/Binding Buffer during an incubation of 10 min at 55 °C. After that, ethanol was added and mixture was applied to the PureLink Spin Column. Centrifugation of 10000 g for 1 min was performed to allow DNA bind to the column, followed by washing with wash buffer and eluting with elution buffer. The purity of the DNA extract was evaluated by spectrophotometer determination of the $A_{260/280}$ ratio, while the concentration was calculated according to the A_{260} .

3.1.8 Bisulfite conversion and Methylation-Specific PCR (MSP)

Genomic DNA was treated using EpiTech Bisulfite Kit (QIAGEN) for complete bisulfite conversion and cleanup for MSP analysis. The bisulfite treatment was conducted following the manufacturer's instructions. 1.5 µg of each DNA sample was used for each

treatment reaction, with 85 μ l Bisulfite Mix, 35 μ l DNA Protect Buffer, and RNase-free water to make up to 140 μ l as the total volume per reaction. After that, a thermal cycler was used to perform the bisulfite DNA conversion according to the following program: 99 °C 5 min, 60 °C 25 min, 99 °C 5 min, 60 °C 85 min, 99 °C 5 min, 60 °C 175 min, and 99 °C hold. Once the bisulfite conversion is complete, Buffer BL was added and the mixture with converted sample and Buffer BL was transferred to the EpiTect column and then centrifuged. After washing with Buffer BW and Buffer BD, converted DNA was eluted from the column by adding Buffer EB and performing centrifugation, and then stored at -80 °C until use.

To examine the methylation status at CpG islands of target genes, MSP was carried out. The bisulfite-treated DNA was amplified with primers for both of the methylated and unmethylated GSTP1 promoters, as described previously [143]. For the methylated GSTP1, primers were: GSTP1 M forward: 5'-TTC GGG GTG TAG CGG TCG TC -3' and GSTP1 M reverse: 5'-GCC CCA ATA CTA AAT CAC GAC G-3'; for unmethylated GSTP1, primers were: GSTP1 U forward: 5'-GAT GTT TGG GGT GTA GTG GTT GTT-3' and GSTP1 U reverse: 5'-CCA CCC CAA TAC TAA ATC ACA ACA-3' [143]. The PCR mixture contained bisulfite-modified DNA 100 ng, 1 $\times$ Platinum Taq buffer, 1.5 mM MgCl₂, 0.24 mM each of dNTP, 0.6 μ M of each primer, and 1 unit of Platinum Taq DNA polymerase (10966034, Invitrogen). The temperature profiles for the amplification were: 95 °C 5 min; 95 °C denaturing 30 sec, 59 °C annealing 45 sec, 72 °C extension 30 sec, for 35 cycles; and a final extension at 72 °C for 10 min. PCR products were analyzed by 3.5% agarose gel with ethidium bromide, as previously described [144].

3.1.9 Bisulfite Genome Sequencing (BGS)

To examine the methylation status at CpG islands of DLEC1 in more details, another methylation analysis assay, bisulfite genome sequencing, was carried out. The bisulfite-treated DNA was amplified for the promoters of interest with BGS primers (Table 3.3). The primers gave fragments within the CpG island in promoter, including 9-19 CpG sites. The temperature profiles for PCR amplification were as follows: 95 °C 5 min, 35 cycles of 95 °C denaturing 30 sec, 50-55 °C annealing 45 sec, 72 °C extension 30 sec, and a final 72 °C extension for 10 min. The amplified fragments were purified with QIAquick PCR Purification Kit (QIAGEN) and cloned into pGEM(R)-T Easy Vector System I (Promega). 4-8 clones of each sample were sequenced for indentifying the methylation status of the contained CpG sites.

Table 3.3 Primers used for BGS

Primer Name	Sequence	Product size
S100A6BGS forward	5'-ATTTTGAGTATTTTTGTGGGATAT-3'	187bp
S100A6BGS reverse	5'-CAACTCAATTTACTCAATTTACTCTTC-3'	
ANXA2BGS forward	5'-GAGTTATTTTTGATTGTTGGGTG-3'	119bp
ANXA2BGS reverse	5'-ACTAACCTAAATAAACTTTTATACCC-3'	
DLEC1BGS1	5'-GAAGATATAAATGTTTATAATGATT-3'	291bp [62]
DLEC1BGS8	5'-ACTATAATAAAAAACCTCAAAATAAA-3'	

3.1.10 Chromatin Immunoprecipitation (ChIP)

ChIP assay was performed with EZ-ChIP kit (#17-371, Upstate, Millipore) to evaluate the histone acetylation level of genes of interest, according to the instruction manual (the detailed manual is available with the kit or online resource provided by the company http://www.komabiotech.co.kr/www/product/pdf/EZ-ChIP_manual.pdf).

Briefly, the adherent cells after different treatments, or transfected with different plasmids, were first crosslinked by adding formaldehyde directly into culture medium to a final concentration of 1% followed by incubation at room temperature for 10 min. After that, 1 ml of 10× Glycine was added to the medium to quench the unreacted formaldehyde. Cells were then washed twice with ice-cold 1×PBS containing protease inhibitors and scraped, collected and pelleted by centrifuging at 4 °C 700 g for 2-5 min. Cell pellets were resuspended in 1 ml of SDS lysis buffer with protease inhibitors and aliquoted 300-400 µl/tube, followed by sonication to shear DNA. The optimal conditions of sonication required for shearing crosslinked DNA to 200-1000 bp in length were needed to be determined for different cell lines. The whole sonication procedure should be carried out on ice (the tube containing samples can be put in ice-water mixture).

After sonication, samples were centrifuged at a minimum of 10000 g but not exceeding 15000 g at 4 °C for 10 min to remove the insoluble materials and then aliquoted to 1×10⁶ cells of lysate for one immunoprecipitation. Each aliquot was diluted to 1 ml with dilution buffer containing protease inhibitors, and precleared by adding 60 µl

of Protein G Agarose 50% slurry by incubating for 1 hr at 4 °C with rotation. Agarose was pelleted by brief centrifugation (3000-5000 g for 1 min), and the 1 ml supernatant was collected into fresh tube, with 10 µl (1%) of the supernatant removed as input control and saved at 4 °C. Immunoprecipitation antibodies ChIP Ab Acetyl-Histone H4 (#17-630, Millipore) and ChIP Ab Acetyl-Histone H3 (#17-615, Millipore) were added to the 1 ml supernatant, and incubated overnight at 4 °C with rotation. After that another 60 µl of Protein G Agarose was added to each IP reaction and incubated for 1 hr at 4 °C with rotation, so as to collect the antibody/histone complex. The protein G agarose/antibody/chromatin complex was pelleted by brief centrifugation and washed by a series of cold washing buffers. The immunocomplexes were then eluted with elution buffer, and DNA was freed from protein/DNA complexes by reverse crosslink. The immunoprecipitated DNA was purified using spin columns and analyzed by PCR (annealing temperature 65 °C) with primers of DLEC1 promoter region, DLEC1ChIPf: 5'-ACAATGACCACAGCGATGAC-3'; DLEC1ChIPr: 5'-GCTGTAGTGGAAGGCCTCAG-3', with a product of 275 bp [141]. Only the promoter with acetylated histone H3/H4 could be amplified by the PCR and result in a band because the immunocomplexes were captured with anti-Acetyl-Histone H3/H4 antibodies.

3.1.11 Reactive Oxygen Species (ROS) analysis

Image-iT LIVE Green Reactive Oxygen Species Detection Kit (I36007, Invitrogen) was used to detect ROS level in live cells affected by different treatments. According to the optimized protocol provided in the kit, oxidatively stressed and non-stressed cells could be reliably distinguished by fluorescence microscopy. In detail, when cells were ready, they were gently washed once with warm HBSS buffer. Then a sufficient amount of 25 μ M carboxy-H₂DCFDA working solution was applied to cover the cells adhering to the plate for an incubation of 30 min at 37 °C protected from light. After that cells were gently washed three times in warm HBSS and some more HBSS buffer added before imaging the cells immediately under fluorescence microscopy. The use of TBHP (also provided in the kit) was employed as a positive control for the induction of ROS, by applying 100 μ M TBHP working solution to the cells and incubating for 90 min (37 °C with 5% CO₂) before the labeling procedures.

3.1.12 Apoptosis analysis (FACS)

Fluorescence Activating Cell Sorter (FACS) was employed to analyze apoptosis of the cells transfected by HBx. Annexin-V-FLUOS Staining Kit (Roche) was used according to the protocols provided, based on Annexin-V-labeling. This kit was used to detect and quantify the apoptosis and differentiate from necrosis at the single cell level. Briefly, Annexin-V-FLUOS labeling solution was prepared by prediluting 20 μ l Annexin-V-Fluos

labeling reagent and 20 μ l Propidium iodide solution in 1ml incubation buffer. 10^6 Cells were trypsinized and washed with PBS and collected by centrifugation. After that cells were resuspended in 100 μ l labeling solution and incubated for 10-15 min at room temperature. 0.5 ml incubation buffer was added to 10^6 cells. The analysis on a flow cytometer was carried out with 488 nm excitation. A 515 nm bandpass filter was used for the fluorescein detection with a filter > 600 nm for PI detection. The results could distinctly differentiate the normal cells (low Annexin, low PI staining), apoptotic cells (high Annexin, low PI staining), and the necrotic cells (high Annexin, high PI staining).

3.1.13 Total protein extraction, protein digestion, and labeling with iTRAQ Reagent for LC-MS/MS

To understand the mechanism behind the epigenetic regulation of HBV in host cells, LC-MS/MS analysis was used to perform a large-scale screening to identify proteins down-regulated in HBV-expressing HepG2.2.15 versus HepG2, and re-expressed by the treatment of 5-Aza-dC (DNMT inhibitor). Detailed procedures were as previously described [2, 145]. Briefly, four days after treatment of 5-Aza-dC, cells were harvested and lysed in 150 μ l of lysis buffer (8 M urea, 0.05% SDS, 4% CHAPS) for 20 min (on ice). Cell lysates were collected by centrifugation and the supernatant with proteins was collected for quantification. The protein concentration was determined by the 2-D Quant Kit (GE Healthcare).

Briefly, a standard curve (the absorbance against the known concentration of Bovine Serum Albumin (BSA)) was plotted using BSA provided in the kit. The protein concentrations in different samples were quantitated with their OD values and the standard curve. After quantification, for each sample, 100 µg protein was precipitated by adding four volumes cold acetone at -20 °C for 2 hr, and then centrifuged at 15000 g for 60 min at 4 °C. After centrifugation, supernatant was removed. The precipitated protein was dissolved with solution buffer then denatured. The cysteines were blocked according to the instructions provided with the kit (Applied Biosystems). 20 µl of 0.25 mg/ml sequence-grade-modified trypsin solution (Promega) was then used to digest each sample at 37 °C overnight. Digested samples were then labeled with the iTRAQ reagents as follows. For Chapter 4, mock-treated HepG2 by DMSO only = iTRAQ 114; 5-Aza-dC-treated HepG2 = iTRAQ 115; mock-treated HepG2.2.15 by DMSO only = iTRAQ 116; 5-Aza-dC-treated HepG2.2.15 = iTRAQ 117. For chapter 5, HepG2 transfected with pXJ40 = iTRAQ 114; HepG2 transfected with pXJ40-HBx A = iTRAQ 115; HepG2 transfected with pXJ40-HBx B = iTRAQ 116; HepG2 transfected with pXJ40-HBx C = iTRAQ 117. To verify that sample preparation techniques do not interfere with digestion and labeling procedures, the BSA standard solution (Pierce) was used in the acetone precipitation, followed by enzymatic digestion with trypsin and labeling with the iTRAQ reagents as described above. These differentially labeled digests were mixed at a ratio of 1:2:1.5:2 and analyzed by LC-MS/MS. [145]

3.1.14 On-line 2-D nano LC-MS/MS analysis

The experiments were performed based on the following equipment settings. One Agilent 1200 nanoflow LC system was interfaced with an electrospray ionization QSTAR XL mass spectrometer (Applied biosystems/MDS Sciex). For the first dimension, 3 μ l combined peptide mixture was loaded onto the Poly-Suifoethyl A SCX column (0.32×50 mm, 5 μ m). The mixture was then eluted stepwise with salt plugs of Buffer D injected. Buffer D consisted of a series of increasing concentration of KCl salt solutions which were contained in ten individual bottles labeling 10, 20, 30, 40, 50, 60, 80, 100, 300, and 500 mM of KCl solution and were used to elute the retained peptides in a stepwise manner from the SCX column by sequential injection.

In the second dimension, with the ten-port valve at position 1, the sequentially eluted peptides were trapped onto the ZORBAX 300SB-C18 enrichment column 1 (0.3×5 mm, 5 μ m). The peptides were washed isocratically by loading buffer A (5% ACN, 0.1% formic acid) at a speed of 0.005 ml/min for 100 min to wash away residual reagent. At one time, peptides bound on the ZORBAX 300SB-C18 enrichment column 2 were eluted by buffer B (0.1% formic acid) and C (a nanoflow gradient of 5–80% ACN + 0.1% formic acid) at 500 nl/min. An increasing concentration of ACN allowed the elution of the concentrated sample. An analytical Zorbax 300SB C-18 reversed-phase column ($75 \mu\text{m} \times 50$ mm, 3.5 μ m) was used for further separation. All columns were used at ambient temperature. After that, with ten-port valve at position 2, column 1 was switched to solvent path of nanopump. The newly eluted peptides would then be trapped by column 2

from SCX. For every experiment, eleven runs were performed.

For electrospray analysis, the HP1200 LC system (Agilent) was interfaced with the QSTAR XL (Applied biosystems/MDS Sciex) MS. Survey scans were acquired from m/z 300-1500 with up to 2 precursors selected for MS/MS from m/z 100-2000 with dynamic exclusion. Fragmentation was achieved by using the rolling collision energy. A cone voltage of 55 V was applied and a high voltage of 2200 V was applied to the spray needle. The curtain gas was N_2 . The same approach applied to other nine runs followed by valve switching, respectively. The four-plex mixture sample was performed again to test the technical reproducibility.

3.1.15 Data analysis and interpretation

Relative abundance quantification as well as the identification of peptide/ protein was carried out by ProteinPilot™ Software v2.0 (Applied biosystems, Software Revision 50861). The MS spectrum were searched for the proteins of *Homo sapiens* according to database uniprot_sprot_20070123, and protein identifications would be accepted based on a ProtScore of more than 2.0, with a confidence value of 99%. All protein identifications made on single unique peptides were discarded. The analysis for the iTRAQ experiments was performed with ProQUANT 1.0. The cut-off for the confidence settings was 75, and the tolerance settings for peptide identification in ProQUANT searches were 0.15 Da for MS and 0.1 Da for MS/MS. All identifications were manually inspected to minimize

machine-related errors. Relative quantification of proteins by using iTRAQ was performed by MS/MS scans. The ratio of areas under the peaks (114, 115, 116 and 117Da) was used for presenting the quantification. 114-117Da indicated the masses of the tags of each iTRAQ reagent. The relative amount of a peptide was calculated by dividing the peak areas of m/z 115.1, 116.1, and 117.1 (labeled samples of interest) by peak area observed at m/z 114.1 (labeled control sample). These calculated ratios were then emended by the overlapping isotopic contributions, and used to estimate the relative abundances of every individual peptide. For proteins with more than one peptide identified, the average peak area ratios (samples compared with control, designated as 115/114, 116/114, and 117/114) were calculated using ratios of all the peptides identified from one protein. The SD, CV for average peak area ratio, and average CV using the CVs for average peak area ratios for each protein were calculated. To account for the differences during the loading of protein, the ratios could be normalized by the overall ratios for all proteins. Protein quantification data with relative expression of > 1.1 or < 0.9 were chosen for further analysis.

3.2 Materials and equipment

3.2.1 Materials, reagents and chemicals

3.2.1.1 Bacteria and Cell lines

The *E. Coli* Strain DH5 α was used for general cloning procedures and plasmid amplification.

Cell lines used in this project include HepG2, Huh7, 293T which were commercially available from ATCC and HepG2.2.15 which was a kind gift from A.P. Lim Seng Gee (NUS, Singapore). HepG2 and Huh7 are both human hepatoma cell lines, and 293T cell line is a variant of HEK293 (derived from a human embryonic kidney), and has high transfection efficiency. HepG2.2.15 is a well established HepG2-derivative cell line, and stably transfected with a plasmid containing 2 head-to-tail dimers of the HBV genome. It can not only release high levels of HBsAg and HBe Ag into supernatants, but also support the assembly and secretion of replicative intermediates of HBV DNA and Dane particles during culture [146], thus it is considered as a good model to study HBV involved modulation of host cells.

3.2.1.2 Kits

For most of the experiments, commercially available kits with high reliability and reproducibility were chosen as follows:

- PureYield Plasmid Midiprep Start-up Kit, A6744, Promega
- QIAprep Spin Miniprep Kit, 27106, QIAGEN
- pGEM-T Easy Vector System I, A1360, Promega
- Nucleofector Cell Line Kit V, VCA-1003, Lonza Cologne AG, Research Biolabs
- Effectene Transfectoin Reagent, 301427, QIAGEN GmbH (Germany), Research Biolabs
- QIAGEN OneStep RT-PCR Kit, 210210, QIAGEN
- iSCRIP 1-st RT-PCR Kit, 1708893, BIO-RAD
- IMx HBsAg Rgnt, REF 2228.21, ABBOTT Laboratories (Singapore) Pte Ltd
- IMx HBsAg Controls, REF 2228.10, ABBOTT Laboratories (Singapore) Pte Ltd
- MEIA 2 Diluent Buffer, 8374.04, ABBOTT Laboratories (Singapore) Pte Ltd
- EZ-ChIP kit, 17-371, Millipore, SciMed Asia Pte Ltd
- EpiTech Bisulfite Kit, 59104, QIAGEN GmbH (Germany), Research Biolabs
- RNeasy Mini Kit, 74104, QIAGEN GmbH (Germany), Research Biolabs
- Rnase Free Dnase set, 79254, QIAGEN GmbH (Germany), Research Biolabs
- Purelink Genomic DNA kit, K182001, Invitrogen
- Annexin-V-FLUOS Staining kit, 11858777001, Roche Diagnostics Asia Pacific Pte Ltd

- Image-iT™ LIVE Green Reactive Oxygen Species Detection Kit, I36007, Invitrogen

3.2.1.3 Other materials and reagents

Other materials and reagents used are:

- Low Molecular Weight DNA Ladder, N3233L, New England Biolabs (USA), Research Biolabs
- 1 kb DNA Ladder, N3232L, New England Biolabs (USA), Research Biolabs
- 100 bp DNA Ladder, N3231L, New England Biolabs (USA), Research Biolabs
- Geneticin (G418), 10131035, Gibco, Invitrogen
- Antibiotic/Antimycotic mixture (100×), 15240062, Gibco, Invitrogen
- Ampicillin ready-made solution, A5354, Sigma-Aldrich
- GoTaq Hot Start Polymerase, M5001, Promega
- Platinum Taq DNA Polymerase, Cat. No. 10966034, Invitrogen
- T4 DNA ligase, M0202L, New England Biolabs (USA), Research Biolabs
- Sss I (CpG) Methylase, M0226S, New England Biolabs (USA), Research Biolabs
- Genomic DNA-Human Adult Normal Tissue: Liver, from a single donor, D1234149-50, BioChain Institute, BST Scientific Singapore
- Genomic DNA-Human Adult Normal Tissue: Liver, female, CP-G02-D1234149-50, BioChain Institute, BST Scientific Singapore
- Total RNA, Lung, Human, 540019, Stratagene (USA), ITS-Science & Medical PTE. LTD.

- Total RNA, Liver, Human, 540017, Stratagene (USA), ITS-Science & Medical PTE. LTD.
- Trypsin EDTA (10×), 15400054, Gibco, Invitrogen
- PBS PH7.2 (1×), 20012050, Gibco, Invitrogen
- Minimum Essential Medium (1×), 11095098, Gibco, Invitrogen
- Dulbecco's Modified Eagle Medium (1×), 11965118, Gibco, Invitrogen
- Polyoxyethylenesorbitan Monolaurate'Elec (Tween 20), P5927, Sigma-Aldrich
- 5-AZA-2'-Deoxycytidine, A3656, Sigma-Aldrich
- Trichostatin A, T8552, Sigma-Aldrich
- 5-Bromo-4-Chloro-3-Indolyl-B-D-Galactopy (X-gal), B9146, Sigma-Aldrich
- Formaldehyde, F. Mol Biology, 47608, Sigma-Aldrich
- Ethanol Absolute, 200 Proof, Molecular, E7023, Sigma-Aldrich
- Glycerol anhydrous, 49780, Sigma-Aldrich
- Ethanol 99% (with 5% methanol), ET099/T18L/A01, AIK MOH PAINTS & CHEMICALS PTE LTD
- Dithiothreitol (DTT), 17-1318-02, GE Healthcare
- Hela Whole Cell Lysate, SC-2200, Santa Cruz, vCell Science
- Rat liver extract, SC-2395, Santa Cruz, vCell Science
- Mouse liver extract, SC-2256, Santa Cruz, vCell Science
- ChIPAb Acetyl-Histone H3, 17-615, Millpore
- ChIPAb Acetyl-Histone H4, 17-630, Millpore
- Monoclonal Anti-beta Actin, A2228, Sigma-Aldrich
- GSTP1 (3F2C2), mouse monoclonal IgG1, SC-66000, Santa Cruz, vCell Science

- CPS1 (N-18), goat polyclonal IgG, SC-10515, Santa Cruz, vCell Science
- Transketolase (N-19), goat polyclonal IgG, SC-46554, Santa Cruz, vCell Science
- Filamin3 (N-16), goat polyclonal IgG, SC-48502, Santa Cruz, vCell Science
- Annexin II (C-10), mouse monoclonal IgG2a, SC-28385, Santa Cruz, vCell Science
- Calcyclin (7D11A8), mouse monoclonal IgG2a, SC-53950, Santa Cruz, vCell Science
- Histone H4 Antibody, 2592, Cell Signaling Technology (USA), Research Biolabs
- Immunopure Peroxidase Conjugated Rabbit anti-goat IgG (H+L), 0031402, Thermo Scientific Pierce, Research Instruments Pte Ltd
- Bradford Dye Reagent, 500-0205, Bio-Rad

3.2.2 Equipment

Equipment used in this project includes:

- Biological safety cabinet, Labconco, Model 362092410900, Gelman Singapore Pet Ltd
- Biological safety cabinet, Labconco, Model LLB-903-B, Gelman Singapore Pet Ltd
- Olympus Microscopy CK×31
- Olympus Fluorescence Microscopy 1×31
- Spectrophotometer: Biospec-mini DNA/RNA/Protein Analyzer, SHIMAZU
- Heidolph Rotomax 120 Orbital Shaker, Colonial Scientific

- Hielscher UP200S ultrasonic processor, Palico Biotech Pte Ltd
- Eppendorf Centrifuge 5702R, B. BRAUN Singapore Pte Ltd
- Sigma 3-16K centrifuge, Palico Biotech Pte Ltd
- Sigma 1-14 centrifuge, Palico Biotech Pte Ltd
- Biometra TGradient Thermocycler, Research Biolabs Pte Ltd
- Eppendorf Mastercycler, B. BRAUN Singapore Pte Ltd
- iQ5 multicolor Real-time PCR Detector System, Bio-Rad Laboratories
- Nucleic acid submerged horizontal electrophoresis, Sub-cell Systems, Bio-Rad
- SYNGENE gel documentation and analysis systems, Insta BioAnalytik Pte Ltd
- Mini-protean Tetra Electrophoresis System, Bio-Rad Laboratories
- Power Pac Basic/Universal power supply, Bio-Rad Laboratories
- Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell, Bio-Rad Laboratories
- Immun-Blot PVDF MEM, 26 cm × 3.3 m, Cat. #1620177, Bio-Rad Laboratories
- CL-Xposure Film (13 × 18 cm), 0034090, Thermo Scientific Pierce, Research Instruments
- Memmert Waterbath, ITS Science & Mediacl Pte Ltd
- Mrc Dry Bath Incubator, Palico Biotech Pte Ltd
- PH Meter, Sartorius Singapore Pte Ltd
- SHIMADZU AW320 Analytical Balance, SHIMADZU
- Rotating Mixer: Agitador Rotativo Orbit, 3000445, JP Selecta (Spain), SciMed Asia Pte Ltd
- Roll and tilt mixer, Movil-Rod, 7000912, SciMed Asia Pte Ltd
- SHEL LAB CO₂ Incubator, Palico Biotech Pte Ltd

- Mrc refrigerated incubator shaker, model LM-570, Palico Biotech Pte Ltd
- SANYO biomedical freezer MDF-U333, MDF-432V, MDF-192, MPR-1013, SCIMED (ASIA) PTE LTD
- Flow cytometry, Beckman coulter
- IMx Automated immunoassay analyzer, No. 8389-59, ABBOTT Laboratories (Singapore) Pte Ltd
- Vacuum Pump, 220 V, 50 Hz, No. 16692, Sartorius Singapore Pte Ltd
- Vortex mixer, Model VM-1000, Mrc, Palico Biotech Pte Ltd
- Amaxa Nucleofetor II, Research Biolabs

Chapter 4 iTRAQ-coupled 2D LC-MS/MS analysis of protein profile associated with HBV-modulated DNA methylation^a

This chapter summarizes results of LC-MS/MS analysis at the proteomic level, to identify new epigenetically suppressed or silenced proteins in HBV-producing HepG2.2.15 cells, and the non HBV-producing HepG2 cells.

4.1 Introduction

The HCC development is believed to be associated with multiple risk factors, including infection by HBV. Based on analysis of individual genes, evidence has indicated the association between HCC and HBV and has also been expanded to epigenetic regulation, with an involvement of HBV in the DNA methylation of the promoter of cellular target genes leading to changes in their expression.

^a Reproduced in part with permission from [Dandan Niu, Jianjun Sui, et al., Proteomics, 9 (2009), 3856-3868.] [1] Copyright [2009] @ Wiley-VCH. Niu Dandan designed the experiments, performed most of the experiments, analyzed the data and wrote the paper. Dr. Sui Jianjun performed the LC-MS/MS machine. Zhang Jianhua helped to analyze the data. Chen Wei Ning supervised the whole project and revised the manuscript.

However, current studies have been largely focused on individual genes regulated epigenetically by HBx, an approach that may be insufficient to precisely define the epigenetic basis of HBV-associated HCC. This feature of present advances on HBV-associated HCC underscores the need to develop a high throughput profiling model to identify new targets of HBV/HBx and to enrich the understanding of the role that HBx plays in epigenetically modulated HCC development.

Proteomic study has been widely used to map a comprehensive protein profile. The traditional proteomic technique, 2-DE, is the most commonly used method to identify differential protein expression on protein mixtures from cellular extracts or tissues [147]. A variety of methods with better sensitivity have been developed using LC-MS/MS to identify large number of proteins from different biological samples. The quantification of proteins in the current proteomics, based on established methods such as growth on isotope-enriched medium [148] and chemical or enzymatic modifications [149], has recently combined iTRAQ and LC-MS/MS [121]. By using this system, we can analyze up to four samples together in each run of the experiment. Different samples are differentially isotopically labeled hence all the derived peptides can get a mass and LC retention time with tag. By collision-induced dissociation (CID) MS/MS analysis, these 4 reporter groups will appear as distinct ions (m/z 114-117). Relative concentration of peptides can be achieved with relative intensities of reporter ions.

In this chapter, by using iTRAQ-coupled 2-D LC/MS-MS analysis, a proteomic profiling was performed to investigate the involvement of HBV in epigenetic gene

regulation. Specifically, we aimed to identify cellular genes down-regulated in HBV-producing HepG2.2.15 cell versus its parent cell HepG2, and those down-regulated genes which could also be re-activated by the treatment of 5-Aza-dC (DNMT inhibitor). A total of 15 proteins were identified by our approach. The significance of these cellular proteins as target of HBV-mediated epigenetic regulation was supported by validation assays, including their re-activation in the cells treated with 5-Aza-dC, real-time RT-PCR and Western blot analysis. The genes encoding two of the proteins, S100A6 and Annexin A2, were further examined for promoter methylation status by bisulfite sequencing. The identified proteins and the DNA methylation status in the promoter of each encoding gene, both could be biomarkers for early diagnosis and even following treatment of HBV-associated HCC.

This approach provides a comprehensive analysis of cellular target proteins to HBV-mediated epigenetic regulation and further analysis should facilitate a better understanding of its involvement in HCC development.

4.2 Results

4.2.1 Validation of HBV genome expression in HepG2.2.15 cell line

HepG2.2.15 is a well established HepG2-derivative cell line, and stably transfected with a plasmid containing 2 head-to-tail dimers of the HBV genome. It not can only release high levels of HBs Ag and HBe Ag into supernatants, but also supports the assembly and secretion of replicative intermediates of HBV DNA and Dane particles during culture [146]. Thus it is considered as a good model to study HBV involved modulation of host cells. The morphology under light microscopy of the two cell lines in normal culture conditions was shown in Fig. 4.1. HepG2.2.15 cells tended to grow in conglobations and colonies (Fig. 4.1A), while HepG2 grew spreading and adhering well to the surface of culture dish (Fig. 4.1B).

Figure 4.1 Morphology of HepG2.2.15 and HepG2 cell lines in normal culture conditions. (A) HepG2.2.15 grew in colonies, more than single layer; (B) HepG2 grew in single layer and adhered well to the surface of culture dish.

To ensure that HepG2.2.15 cell line was indeed HBV-producing, the HBV genome expression was examined. The secretion of Hepatitis B Surface Antigen (HBs Ag) in the culture medium of HepG2.2.15 was detected by using IMx immunoassay system (Abbott, USA) and IMx HBs Ag Kit (Abbott, USA), a qualitative microparticle enzyme immunoassay (MEIA) for the detection of HBsAg. Results from IMx analysis shown in Fig. 4.2A indicated that, HBsAg was detected in culture medium of HepG2.2.15 cells but not in that of HepG2 cells. In addition, RT-PCR results shown in Fig. 4.2B indicated that mRNA of both HBs Ag and HBV core antigen (HBc Ag) were detected in HepG2.2.15 but not in HepG2 cells. As a positive control, the internal expressed β -actin was detected in both types of the cells. These results confirmed that HepG2.2.15 cell line was indeed HBV-producing.

Figure 4.2 Validation of HBV genome expression in HepG2.2.15. (A) IMx analysis of HBs Ag secreted in cell culture medium of both HepG2 and HepG2.2.15. The positive control and negative control are reagents provided in the IMx kit. (B) RT-PCR performed to show the HBsAg and HBc Ag expression in the mRNA of both of the cell lines. The negative control was no template DNA added but equal volume of water instead.

4.2.2 iTRAQ analysis of the differentially expressed proteins in HepG2.2.15 and HepG2

To investigate the underlying mechanism of epigenetic regulation of HBV in the host cells, we used LC-MS/MS analysis to identify genes down-regulated in HepG2.2.15 versus HepG2, and those which were re-activated after the treatment of 5-Aza-dC (DNMT inhibitor). The proposed strategy was illustrated in Fig. 4.3: **n1** indicated the number of proteins down-regulated in HBV-producing HepG2.2.15 as compared with HepG2; **n2** indicated the number of proteins reactivated in HepG2.2.15 after the 5-Aza-dC treatment. This approach should enable us to identify targets involved in HBV mediated epigenetic gene regulation, in particular the intersection of **n1** and **n2** (represented by **N**).

Figure 4.3 Identification of genes reactivated by 5-Aza-dC in HepG2.2.15 by LC-MS/MS analysis. **n1**: the number of proteins down-regulated in HBV-producing HepG2.2.15 as compared with HepG2; **n2**: the number of proteins reactivated in HepG2.2.15 after the 5-Aza-dC treatment. **N**: the intersection of **n1** and **n2**, proteins down-regulated by HBV via DNA hypermethylation.

Three independent runs were carried out. A total of 167 proteins were detected in one typical run, with 38 proteins (**n1=38**) down-regulated in HBV-producing HepG2.2.15 compared with HepG2. On the other hand, 54 proteins (**n2=54**) were up-regulated in

HepG2.2.15 after the 5-Aza-dC treatment, suggesting that these coding genes may be inhibited by DNA methylation. Interestingly, 22 proteins were found overlapping (N=22) which may well be the candidate targets of HBV-associated DNA methylation-regulated proteins. The identification of proteins with significant changes in their expression level was based on the ProtScore with a cut-off at 2.0 (confidence value of 99%). Combined with the results from three separate experiments, fifteen unique proteins were identified based on the above criteria and consistent with the repeats. One representative MS/MS spectrum for a peptide of ANXA2 is shown in Fig. 4.4. The mass data are shown in Table 4.1 and Table 4.2.

Figure 4.4 One representative MS/MS spectrum indicating one peptide, SYSPYDMLESIR, of Annexin A2. The ion assignments are: HepG2 mock treated = iTRAQ 114; HepG2 5-Aza-dC treated = iTRAQ 115; HepG2.2.15 mock treated = iTRAQ 116; HepG2.2.15 treated by 5-Aza-dC = iTRAQ 117.

Table 4.1 Representative MS/MS peptide sequences of some of the identified proteins

Unused	%Cov	Accessions	Contrib	Conf	Sequence
Carbamoyl-phosphate synthase					
12.74	37.93	CPSM_HUMAN (P31327)	2.00	99	AQTAHIVLEDGTK
12.74	37.93	CPSM_HUMAN (P31327)	2.00	99	FLGVAEQLHNEGFK
12.74	37.93	CPSM_HUMAN (P31327)	2.00	99	FVHDNYVIR
12.74	37.93	CPSM_HUMAN (P31327)	2.00	99	SIFSAVLDELK
12.74	37.93	CPSM_HUMAN (P31327)	2.00	99	VSQEHPVVLTk
Histone H4					
11.53	54.37	H4_HUMAN (P62805)	2.00	99	DAVTYTEHAK
11.53	54.37	H4_HUMAN (P62805)	2.00	99	DNIQGITK
11.53	54.37	H4_HUMAN (P62805)	2.00	99	DNIQGITKPAIR
11.53	54.37	H4_HUMAN (P62805)	2.00	99	ISGLIYEETR
11.53	54.37	H4_HUMAN (P62805)	2.00	99	VFLENVIR
Annexin A2					
8.79	41.00	ANXA2_HUMAN (P07355)	2.00	99	GDLENAFLNLVQCIQNKPL LYFADR
8.79	41.00	ANXA2_HUMAN (P07355)	2.00	99	GVDEVTIVNILTNR
8.79	41.00	ANXA2_HUMAN (P07355)	1.70	98	SALSGHLETVILGLLK
8.79	41.00	ANXA2_HUMAN (P07355)	1.52	97	SYSPYDMLESIR
Transketolase					
8.27	37.08	TKT_HUMAN (P29401)	2.00	99	HQPTAIIAK
8.27	37.08	TKT_HUMAN (P29401)	2.00	99	NMAEQIIQEIYSQIQSK
8.27	37.08	TKT_HUMAN (P29401)	2.00	99	SVPTSTVFYPSDGVATEK
Filamin B					
7.01	23.17	FLNB_HUMAN (O75369)	2.00	99	LIALLEVLSQK
7.01	23.17	FLNB_HUMAN (O75369)	2.00	99	VNIGQGSHPQK
7.01	23.17	FLNB_HUMAN (O75369)	2.00	99	VVASGPGLEHGK
7.01	23.17	FLNB_HUMAN (O75369)	1.52	97	TVTAMDVVYALK
Protein S100-A6					
5.44	47.78	S10A6_HUMAN (P06703)	2.00	99	ACPLDQAIGLLVAIFHK
5.44	47.78	S10A6_HUMAN (P06703)	2.00	99	LQDAEIAR

Table 4.2 List of differentially expressed cellular proteins

Protein name	Accession	Protein score	116 : 114 ($\pm$ SD) ^{a)}	117 : 116 ($\pm$ SD) ^{b)}	Function
Signaling regulation proteins					
Protein S100-A6	P06703 S10A6	5.44	0.836 $\pm$ 0.093	1.073 $\pm$ 0.164	Calcium binding
Annexin A2	P07355 ANXA2	5.19	0.575 $\pm$ 0.041	1.242 $\pm$ 0.297	Calcium-depend ent phospholipid- binding
Serotransferrin	P02787 TRFE	3.36	0.959 $\pm$ 0.143	1.626 $\pm$ 0.134	Iron binding transport
Protein S100-A4	P26447 S10A4	3.05	0.447 $\pm$ 0.076	1.023 $\pm$ 0.109	Calcium binding
Eukaryotic translation initiation factor 5A-2	Q9GZV4 IF5A2	2.14	0.867 $\pm$ 0.002	1.067 $\pm$ 0.116	Eukaryotic translation initiation factor
Metabolic enzymes					
Carbamoyl-phosph ate synthase	P31327 CPSM	12.74	0.407 $\pm$ 0.153	1.127 $\pm$ 0.057	Involved in the urea cycle in removing excess ammonia
Transketolase	P29401 TKT	8.27	0.640 $\pm$ 0.002	1.235 $\pm$ 0.124	Transketolase activity
L-lactate dehydrogenase B chain	P07195 LDHB	5.59	0.661 $\pm$ 0.032	1.436 $\pm$ 0.234	Dehydrogenation of lactate
Triosephosphate isomerase	P60174 TPIS	5.02	0.884 $\pm$ 0.031	1.144 $\pm$ 0.051	Triosephosphate isomerase activity
Peroxiredoxin-6	P30041 PRDX 6	2.67	0.735 $\pm$ 0.153	1.219 $\pm$ 0.130	Redox regulation of the cell
ATP-dependent RNA helicase DDX3X	O00571 DDX3 X	2.01	0.790 $\pm$ 0.090	1.640 $\pm$ 0.106	ATP-dependent RNA helicase
Structural proteins					
Histone H4	P62805 H4	11.53	0.726 $\pm$ 0.055	1.519 $\pm$ 0.475	Core component

Filamin-B	O75369 FLNB	7.01	0.749±0.023	1.45±0.466	of nucleosome Connecting cell membrane constituents to the actin cytoskeleton Branching of actin filaments and linking actin filaments to membrane glycoproteins
Filamin-A	P21333 FLNA	2.81	0.771±0.044	1.637±0.024	
40S ribosomal protein S3a	P61247 RS3A	2.0	0.696±0.079	1.605±0.165	Ribosomal protein

a) 116:114, the ratio of protein expression level in mock treated HepG2.2.15 (116) to mock treated HepG2 (114).

b) 117:116, the ratio of protein expression level in 5-Aza-dC treated HepG2.2.15 (117) to mock treated HepG2.2.15 (116).

These 15 proteins, which were down-regulated in HepG2.2.15 compared with HepG2, and can further be re-activated by 5-Aza-dC treatment, were subsequently categorized into separate clusters (Table 4.2) according to their cellular functions suggested by the online Swiss-Prot database. As shown in Table 4.2, differentially expressed proteins included five signaling regulation proteins, six metabolic enzymes and four structural proteins. In the category of signaling proteins, three of them (Protein S100-A6 and A4, Annexin A2) were calcium binding proteins. Two of the S100 proteins were significantly down-regulated in HepG2.2.15 compared with HepG2 (116:114), although the up-regulation after 5-Aza-dC treatment was not as significant (117:116). On the other hand, Annexin A2 showed a decreased level in HepG2.2.15 and a significantly increased

level in HepG2.2.15 after 5-Aza-dC treatment. The other two proteins, serotransferrin and eukaryotic translation initiation factor 5A-2, showed notable down-regulation in HepG2.2.15 while the up-regulation after treatment was not as significant. Finally, metabolic enzymes and structural proteins showed a decreased level in HepG2.2.15 and a significantly increased level in HepG2.2.15 after 5-Aza-dC treatment.

4.2.3 Real-time RT-PCR analysis for differentially expressed proteins

To assess if the changes of the protein expression were significant, genes from each of the clusters listed in Table 4.2 were analyzed at mRNA level by real-time RT-PCR.

As shown in Fig. 4.5, S100A6, ANXA2, His H4 and FLNB (panel A, B, D & E), presented a consistent expression pattern with that identified in the LC-MS/MS analysis. Specifically, their expression was reduced in HepG2.2.15 (column 3) compared with HepG2 (column 1), while this reduction in HepG2.2.15 could be partially rescued after the 5-Aza-dC treatment (column 4). For TKT and CPS1 (Figure 4.5C & F), although the decrease of their expression was greatly significant in HepG2.2.15 compared with HepG2, the 5-Aza-dC treatment seemed to have little effect on the re-activation after the treatment.

Figure 4.5 Representative results of real-time RT-PCR. Real-time RT-PCR showing the expression of several proteins at the mRNA level in HepG2 and HepG2.2.15 (column 1 and 3), and both of them treated by 5-Aza-dC (column 2 and 4). (A) Protein S100-A6 (S100A6); (B) Annexin A2 (ANXA2); (C) Transketolase (TKT); (D) Histone H4 (His H4); (E) Filamin-B (FLNB); (F) Carbamoyl-phosphate synthase (CPS1). Three independent experiments were carried out. Expression levels of the protein normalized to HepG2 (the HepG2 level was set as 1) were indicated by “*”: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; while expression normalized to HepG2.2.15 was indicated by “#”: #, $P < 0.05$.

4.2.4 Western blot analysis of differentially expressed proteins

To extend our validation, Western blot analysis was performed for S100A6, ANXA2, CPS1, TKT and FLNB (Fig. 4.6). Results in Fig. 4.6A showed that S100A6 was largely depleted in HBV-producing HepG2.2.15 cells (lane 3) compared with its level in HepG2 cells (lane 1). The treatment of DNMT1 inhibitor 5-Aza-dC resulted in little changes in the level of S100A6 in HepG2 cells (lane 2), but its level was significantly increased in HepG2.2.15 after the same treatment (lane 4). This result suggests that S100A6 expression may be inhibited by HBV genome expression, possibly as a result of DNA methylation in the S100A6 promoter. Similar result was obtained for ANXA2 (Fig. 4.6B) and FLNB (Fig. 4.6E), with the only difference that the expression of these proteins in HepG2.2.15 was strongly reduced (Fig. 4.6B, lane 3; Fig. 4.6E, lane 3), not completely inhibited as that of S100A6 in HepG2.2.15 (Fig. 4.6A, lane 3).

Figure 4.6 Western blot analysis of protein expression in both of HepG2 and HepG2.2.15 with or without treatment of 5-Aza-dC. (A) S100A6 expression; (B) ANXA2 expression; (C) CPS1 expression; (D) TKT expression; (E) FLNB expression. Up panel shows the expression of S100A6, ANXA2, CPS1, TKT or FLNB; while the bottom panel gives β-actin which was used as loading control. Lane 1 and 2 are HepG2 without or with 5 μM 5-Aza-dC treatment for 96 hr; lane 3 and 4 are HepG2.2.15 with or without 5-Aza-dC treatment.

On the other hand, results of CPS1 in Western shown in Fig. 4.6C were consistent with the real-time RT-PCR analysis (Fig. 4.5F), indicating that CPS1 was undetected in HepG2.2.15 as compared with its level in HepG2. In addition, the 5-Aza-dC treatment showed little effect on its expression level. And similar result was shown for TKT (Fig. 4.6D).

4.2.5 DNA methylation analysis on S100A6 and ANXA2

To investigate whether the expression alteration was caused by DNA methylation, the Bisulfite Genomic Sequencing was carried out to assess the promoter DNA methylation status of S100A6 and ANXA2. The widely used bisulfite conversion of DNA will change the unmethylated cytosine (C) into uracil which would be presented as thymine (T); but the methylated cytosine remains cytosine. Therefore, if the sequencing result of a CpG site is CG, the original sequence should be methylated-Cytosine; while if the result is TG, the original sequence must be unmethylated-Cytosine. This will enable us to identify the methylation status of the CpG sites in the target promoters [131]. Promoters from both HepG2 and HepG2.2.15 cells were analyzed after the treatment of 5-Aza-dC or mock treatment (see Materials and Methods). Fig. 4.7A showed that the 7.89% of S100A6 promoter in mock treated HepG2 cells was methylated (row 1), while in mock treated HepG2.2.15 the percentage of methylation sites was significantly higher at 43.18% (row 3). This result was consistent with the expression pattern of S100A6 in both HepG2.2.15 and HepG2 cells, namely real-time RT-PCR in Fig. 4.5A and Western blot analysis in Fig.

4.6A. Taken together, our data suggested that S100A6 was expressed at a higher level in HepG2 as a result of lower methylation in its promoter. In contrast, the expression of S100A6 was significantly inhibited in HepG2.2.15 in which its promoter was significantly higher methylated, likely due to the active HBV replication in these cells.

Figure 4.7 Promoter methylation analysis by genomic bisulfite sequencing of S100A6 and ANXA2 5' CpG sites in both HepG2 and HepG2.2.15 with or without treatment of 5-Aza-dC. (A) 11 CpG dinucleotides were analyzed in the 5' promoter of S100A6 (represented as 11 columns). 4-8 clones of each sample were sequenced and the results were presented as follow: Open area indicates the percentage of unmethylated CpG sites, while dark area indicates percentage of methylated CpG sites. The quantitative result in percentage of methylated CpG sites was listed on the right of the column groups. Three independent experiments were carried out and representative result from one of

them was listed here. (B) 9 CpG dinucleotides were analyzed in the 5' promoter of ANXA2. (C) Representative bisulfite sequencing results of the S100A6 promoter region showing the CpG methylation status of bisulfite treated DNA from two cell lines mock treated or 5-Aza-dC treated. Rectangles indicate the methylated CpG sites, while ellipses for unmethylated CpG sites. Arrows mark the CpG sites whose methylation status was changed by 5-Aza-dC treatment.

The correlation between the expression level of S100A6 and the extent of the methylation in its promoter was also supported by the treatment of both HepG2.2.15 and HepG2 cells by 5-Aza-dC, a DNMT inhibitor. Specifically, the methylation of the S100A6 promoter in HepG2 cells was reduced from 7.89% to 2.27% (row 2, Fig. 4.7A) while it was decreased from 43.18% to 30.30% in HepG2.2.15 cells (row 4, Fig. 4.7A). This result provided further evidence that the reduction of the expression of S100A6 in HepG2.2.15 compared with HepG2 was a result of DNA methylation in its promoter.

The same methylation analysis was also performed for ANXA2, as shown in Fig. 4.7B. The difference of promoter DNA methylation status in both HepG2 and HepG2.2.15 was greatly significant that the percentage of methylation was only 2.78% in HepG2 (row 1, Fig. 4.7B) but reached to a high percentage of 97.22% in HepG2.2.15 (row 3, Fig. 4.7B). This result was well correlated to the significant down-regulation of ANXA2 expression in HepG2.2.15 compared with HepG2, detected by both real-time RT-PCR (Fig. 4.5B) and Western blotting (Fig. 4.6B). And similarly to S100A6, the percentage of methylation showed certain decrease in both of the cells after 5-Aza-dC treatment: 2.78% to 0% in HepG2 (row 1-2, Fig. 4.7B) and 97.22% to 80.56% in HepG2.2.15 (row 3-4, Fig. 4.7B); and this alteration could also support the increase of ANXA2 expression in HepG2.2.15

after 5-Aza-dC treatment (Fig. 4.5B, Fig. 4.6B).

Fig. 4.7C provides representative sequencing data of the S100A6 promoter region of -130 ~ -113 (transcription starting site as +1) including two CpG sites as indicated, and showing the CpG methylation status of bisulfite treated DNA from two cell lines mock treated or 5-Aza-dC treated. As shown in the upper row, HepG2 was partially methylated (Fig. 4.7C, upper left panel) while after the treatment (Fig. 4.7C, upper right panel) of 5-Aza-dC, the methylated site (rectangle in left panel with arrow) became unmethylated (ellipse in right panel with arrow). In the lower row, two CpG sites shown were both methylated in HepG2.2.15 (lower left panel), and after 5-Aza-dC treatment, both of them were demethylated (lower right panel).

4.3 Discussion

HCC remains to be one of the most aggressive malignancies in human and is closely associated with chronic hepatitis B and C virus infection [11]. However, the underlying mechanisms of the HBV involved malignant transformation of infected cells remain unclear.

Aberrant DNA methylation has been shown to affect gene expression and chromatin stability on the transcriptional level, and attributed as an important epigenetic alteration in HCC onset. In recent years, HBV infection has also been associated with DNA hypermethylation of tumor suppressor genes and thus leading to increased tumor risk. This is because the HBV-mediated hypermethylation of TSGs can suppress or silence TSG expression. The reported studies have however been limited by the fact that specific protein are individually analyzed, thus a comprehensive understanding is missing. This limitation can be overcome by a proteomics study to map a comprehensive protein profile. Using a proteomic approach, we aimed to identify target proteins whose regulation is influenced by HBV infection via epigenetic regulation.

To this end, two cell lines HepG2 and HepG2.2.15 were used in our approach, HepG2.2.15 being a HepG2-derivative cell line which not only releases high levels of HBsAg and HBe Ag into supernatants, but also support the assembly and secretion of replicative intermediates of HBV DNA and Dane particles [146]. The expression of HBV

in the HepG2.2.15 cells we used in our lab was solidly confirmed (Fig. 4.2) and this provided the basis of analysis on HBV associated alterations of proteins in HepG2.2.15 compared with HepG2.

As compared with the traditional proteomic methods such as 2-DE gel analysis followed by MS/MS identification, our iTRAQ-coupled 2-D LC-MS/MS approach provides higher detection sensitivity and holds the promise of effectively depicting cellular protein profile in response to various treatments. From three independent experiments, fifteen proteins were consistently identified (Table 4.2) as being down-regulated in HepG2.2.15 cells compared with their expression level in HepG2 cells. The level of these proteins was then found to be up-regulated in HepG2.2.15 cells after the treatment of 5-Aza-dC, a DNA methyltransferase inhibitor which reduces the extent of DNA methylation. These fifteen proteins were then classified by three groups for their functions (Table 4.2), and validation of the changes in the expression level was carried out on a number of them. The validation results by real time RT-PCR (Fig. 4.5) on mRNA level were overall consistent with that in LC-MS/MS results. Five of them, S100A6, ANXA2, CPS1, TKT and FLNB, were further confirmed by Western blot on the protein level (Fig. 4.6).

In the group of signaling regulation proteins, loss of ANXA2 has been reported in early onset of tumorigenesis in esophageal and prostate carcinoma [150, 151]. Another report has indicated that its expression is reduced or lost in prostate cancer cells and its re-expression inhibits prostate cancer cell migration [151, 152]. In the context of HCC,

some pieces of evidence have suggested that ANXA2 can be up-regulated in higher metastatic HCC cell lines [117], and over-expressed in malignant hepatocytes in HCC [22, 153]. These published results indicated that ANXA2 may play different roles in different human cancers, acting as a tumor suppressor gene in prostate cancer while promoting tumorigenesis in HCC. Our results showed that ANXA2 was down-regulated in HBV-producing HepG2.2.15 cells compared with HepG2 cells. This discrepancy with the published data on its expression in HCC may be explained by the replication state of HBV which is high in HepG2.2.15 but low in HCC cells. It remains to be determined whether other HBV proteins are involved in the changes in the expression level of ANXA2, as reported for HBV DNA polymerase which indirectly interacts with ANXA2 [154].

Another protein of interest is serotransferrin. This protein is involved in the iron transportation from absorption sites and stimulation of cell proliferation. Clinical study has indicated that the serum transferrin level was significantly lower in HBV carriers and HBV-positive HCC patients, compared with healthy individuals [125]. The decrease of serotransferrin expression in HepG2.2.15 cells compared with the level in HepG2 cells was consistent with the reported findings, and further suggested that its down-regulation may be resulted from epigenetic regulations including HBV-induced DNA methylation.

The protein of particular interest, which might be regulated by HBV through DNA hypermethylation, is S100A6. S100A6 (calcyclin) is one of the members in S100 family which includes a group of low molecular weight (9-13 kDa) calcium binding proteins with two EF-hand motifs. Proteins in this family are thought to activate target proteins and

therefore influence cellular response along the calcium signal transduction pathway [155-157]. Individual S100 proteins may have specific functions in different cell types. For example, S100A6 has been considered to be involved in the cell proliferation and differentiation, and especially involved in invasion and metastasis of cancer cells. It has been reported that S100A6 expression is increased in human colorectal adenocarcinoma [158], while absent in prostate cancer [159] and medulloblastoma [160], due to tumor-specific hypermethylation. In the case of HCC, clinical studies have reported that S100A6 can be detected in cholangiocarcinoma [60], liver metastasis and colorectal carcinoma [161], but rarely or weakly detected in HCC. In this study, we identified from the proteomics analysis that S100A6 was down-regulated in HBV-producing HepG2.2.15 cells compared with the level in HepG2 cells. This inhibition was slightly reversed by 5-Aza-dC treatment. The up-regulation of S100A6 in HepG2.2.15 after 5-Aza-dC treatment, while first detected in the LC-MS/MS analysis, was further confirmed by real-time RT-PCR (Fig. 4.5A) and Western blot analysis (Fig. 4.6A). Furthermore, the BGS analysis in the S100A6 promoter also revealed that the alteration of S100A6 expression in HepG2.2.15 was due to DNA methylation (Fig. 4.7). Taken together, these results suggest that the expression of S100A6 might be affected by HBV through DNA methylation in its promoter region. Interestingly, our findings were also supported by two recent reports showing that S100A6 is down-regulated in HepG2.2.15 [162], which could due to hypermethylation by HBx and its expression can be re-activated by 5-Aza-dC treatment [114].

Two of the more significant proteins in the group of metabolic enzymes included

transketolase (TKT) and Carbamoyl-phosphate synthase (CPS1). TKT, a Ca^{2+} -binding thiamin-dependent enzyme, is mainly responsible for ribose synthesis. Stimulators of transketolase enzyme synthesis such as thiamin (vitamin B-1) support a high rate of nucleic acid ribose synthesis necessary for tumor cell survival, chemotherapy resistance, and proliferation [116], although its role in HCC remains to be elucidated. Our LC-MS/MS results indicated that TKT was down-regulated in the HBV-producing HpeG2.2.15 cells compared with HepG2 cells and its level was slightly up-regulated after 5-Aza-dC treatment. It is possible that HBV replication in HepG2.2.15 inhibits TKT expression, resulting to a compromised cellular nutrition and protection in the normal liver and thus contributing to the onset of HCC. The second protein, CPS1 is involved in the urea cycle and is very important for getting redundant ammonia out of the cell. Clinical report has indicated that the CPS1 is under-expressed significantly in primary HCC tissues [163], although the underlying mechanism remains poorly understood. This protein was found in our investigation to be dramatically suppressed in HepG2.2.15 cells compared with HepG2 cells, suggesting that HBV replication in HepG2.2.15 cells may be involved in its suppression. However, the increase of its expression in HepG2.2.15 after 5-Aza-dC treatment was not as significant in real-time RT-PCR (Fig. 4.5F) and Western blot analysis (Fig. 4.6C). The possible explanation might be that the down-regulation of CPS1 was not resulted by DNA methylation but by other mechanisms. Interestingly, our hypothesis was indirectly supported by the search of CpG islands in the promoter of CPS1 using an online program EMBOSS CpGPlot, which showed the absence of CpG islands in its promoter (Fig. 4.8). Since DNA hypermethylation regulates gene expression on the transcriptional level through the CpG islands in the promoter, it was likely that CPS1 gene

expression without CpG islands in its promoter was regulated by other mechanisms.

Figure 4.8 EMBOSS CpGPlot results of CPS1 promoter. A fragment of 2150 bp including the 2 kbp promoter and the first 150 bp of mRNA of CPS1 was plotted. The first panel indicates Observed/Expected ratio of CpG sites; the second panel indicates percentage of C+G; and the last panel indicates the identified CpG island. A CpG island will be identified if the O/E ratio > 0.6; percentage of C+G > 50%, and total length > 100 bp.

Histone H4, in the group of structural proteins, is one of the core components of nucleosome enabling it to play a crucial role in a variety of cellular machineries including transcription regulation, DNA replication and repair, as well as chromosomal stability. Most studies of histone H4 have been on its post-translational modifications such as histone acetylation, methylation, phosphorylation, and ubiquitination. Our LC-MS/MS results have indicated that the expression of histone H4 could also be regulated at the

transcription level by HBV-mediated DNA methylation.

In conclusion, we compared the proteome expression pattern of HBV-producing HepG2.2.15 cells and HepG2 cells, as well as both of the cells treated by DNMT inhibitor 5-Aza-dC. Our findings suggested that HBV replication in HepG2.2.15 cells affected the level of a wide range of cellular proteins possibly involving epigenetic regulation via DNA methylation of the promoter of target genes. The identification of 15 proteins provided molecular clues on clinical observations related to HBV-induced HCC. And due to one of its characteristics that DNA methylation usually occurs in the early stage of cancer development, the hypermethylation of these genes could serve as candidate biomarkers for clinical studies and help in early scanning and diagnosis in populations with higher risks. Further detailed proteomics analysis coupled with validation assays should shed new lights on the mechanisms of epigenetic regulation of HBV-mediated hepatocellular carcinogenesis.

Chapter 5 iTRAQ-coupled 2D LC-MS/MS analysis of protein profile in cells transfected with genotype specific HBx^b

Results in Chapter 4 focused mainly on identifying target proteins which were epigenetically modulated by HBV replication. This chapter summarized results of the proteomics analysis in cells expressing HBx. Down-regulated proteins identified by this approach may be targets for further analysis for their role in HBx-modulated epigenetic changes.

5.1 Introduction

In addition to the proteomic analysis of cellular target proteins regulated by HBV-mediated DNA hypermethylation, the role of HBx in this process was also interesting, because of the four proteins encoded by HBV genome, HBx has been the only protein that was found to epigenetically inhibit TSGs like E-cadherin [21, 59] and p16^{INK4a} [20, 71, 72]. The HBx mediated epigenetic regulation of target promoters involves deregulation of DNA methyltransferases (DNMTs), resulting in silencing of TSG and development of HCC [22].

^b Reproduced in part with permission from [Feng Huixing, Li Xi, Niu Dandan et al., Journal of Proteomics, E-pub ahead of print, PMID: 20026004] Copyright [2010] @ Elsevier. Work described in this chapter was a collaborative work. The LC-MS/MS, real-time RT-PCR and Western data were generated by Dr. Feng Huixing. Niu Dandan analyzed and discussed the data in this chapter focusing on down-regulated proteins by HBx as potential targets of HBx-induced DNA methylation, as a complementary part to for HBV-induced DNA methylation discussed in Chapter 4.

On the other hand, HBV can be classified into eight genotypes from A to H based on 8% DNA sequence divergence [24]. Earlier studies have indicated a clinical linkage between serotypes and vaccine escape mutants [164]. It has also been suggested that particular HBV genotypes may correlate with differences in clinical features of viral infection [51, 165]. Preliminary investigation by our lab using 2-D gel coupled with MS identification has yielded promising indications on the genotype-associated protein profile in HBx producing cells [36]. However, the technique is not as sensitive and fast as compared with LC-MS/MS analysis which has been applied in the work presented in Chapter 4.

To this end, the HBx-induced protein profiles in HepG2 cells were established by iTRAQ-coupled 2-D LC-MS/MS proteomic analysis. The differences in protein expression levels of HepG2 cells and those transfected with HBx of three different genotypes (A, B and C) were revealed and quantified. Eleven proteins were found to be down-regulated in HepG2 cells transfected by HBx compared with cells transfected by empty vector, while 13 others up-regulated. Interestingly, a number of the proteins were also identified in our comprehensive proteomic analysis between HepG2 and HBV-expressing HepG2.2.15 (Chapter 4). These include noticeably cytoskeleton proteins, metabolic enzymes and signal transduction proteins. Alterations of expression level in some of the genes have been linked to cancer development, suggesting their tumor suppressor characteristics.

Taken together, a cell culture based proteomic analysis was established to understand

the early cellular response to genotype specific HBx and our data therefore revealed a specific role of HBx in the inhibition of cellular targets. Considering its reported involvement in DNA hypermethylation and therefore inhibition of TSGs, the results in this chapter, combined with Chapter 4, should provide helpful information of candidate target proteins for further mechanism and functional studies on HBx mediated DNA methylation in HCC development.

5.2 Results

5.2.1 HBx expression in transfected cells

HBx of different genotypes from clinical samples were cloned into high mammalian expression vector pXJ40 and confirmed by sequencing. To mimic the early HBV infection and characterize cellular response to HBV infection specifically associated with HBx, HepG2 cells were transfected with HBx of genotypes A/B/C as well as empty vector pXJ40. The expression of HBx was confirmed by Western blot using anti-HA antibody (Fig. 5.1), as HA tag was fused in-frame with the genotype-specific HBx. The result showed that the HBx of three genotypes was expressed in HepG2 cells.

Figure 5.1 Expression of HBx A/B/C verified by Western Blot. HBx (17.5 kDa) expression was detected by HA tag contained in the vector pXJ40. pXJ40 was also included as an empty vector control, and β -actin was also detected as the internal control with a size of 42 kDa.

5.2.2 iTRAQ analysis of differentially expressed proteins

The comprehensive protein profile showing global cellular events in response to genotype specific HBx can then be analyzed using the high throughput, broad-spectrum and sensitivity proteomic method iTRAQ-coupled 2-D LC-MS/MS.

To establish the protein profiles in the HepG2 cells transfected with HBx compared with empty vector pXJ40, the iTRAQ was used to label as following: empty vector pXJ40 = iTRAQ 114; HepG2 transfected with pXJ40-HBx genotype A = iTRAQ 115; HepG2 transfected with pXJ40-HBx genotype B = iTRAQ 116; HepG2 transfected with pXJ40-HBx genotype C = iTRAQ 117. In each of the three independent experiments, the total proteins were extracted from the cells after transfection and labeled with iTRAQ reagents with the same assignments of the isobaric ions. ProQUANT was used to identify and quantify the different protein expression levels among different samples.

Totally there were 163 proteins identified from 3 independent experiments as

mentioned above. The standard errors shown in Table 5.1 were generated by three independent experiments. Identification and quantification proteins with significant change of expression level were based on the ProtScore with the Unused protein score more than 2.0. This gave the confidence value of 99% and peptide hits per protein at confidence not less than 95% per peptide. Other proteins identified with ProtScore less than 2.0 were not included for further analysis. Among the 163 identified proteins, 24 differentially expressed proteins were listed here and classified as 4 major groups according to their functions. 5 metabolic proteins, 10 signaling proteins, 6 cytoskeletal proteins and 3 cell growth related proteins were shown in Table 5.1.

Table 5.1 List of differentially expressed cellular proteins in HBx transfected HepG2 cells

Protein name	Accession	Protein score	115 : 114 ($\pm$ SD) ^{a)}	116 : 114 ($\pm$ SD) ^{b)}	117 : 114 ($\pm$ SD) ^{c)}	Function
Signaling regulation proteins						
Annexin A2	P07355 ANXA2	6.38	1.33 $\pm$ 0.24	1.12 $\pm$ 0.01	1.08 $\pm$ 0.01	Calcium binding
hnRNP A2 / B1	P22626 ROA2	6.01	0.83 $\pm$ 0.02	0.94 $\pm$ 0.05	0.93 $\pm$ 0.07	pre-mRNA processing
Protein S100-A6	P06703 S10A6	5.22	0.90 $\pm$ 0.10	0.89 $\pm$ 0.04	0.84 $\pm$ 0.05	Calcium-binding
Protein S100-A11	P31949 S10AB	3.85	0.94 $\pm$ 0.04	0.86 $\pm$ 0.10	0.86 $\pm$ 0.02	Calcium-binding
hnRNP A1	P09651 ROA1	3.45	0.94 $\pm$ 0.02	0.93 $\pm$ 0.04	1.00 $\pm$ 0.05	Packaging of pre-mRNA into hnRNP particles, may modulate splice site selection
hnRNP U	Q00839 HNRPU	2.94	1.28 $\pm$ 0.11	1.30 $\pm$ 0.02	1.19 $\pm$ 0.05	pre-mRNA binding; Binds to double- and single-stranded DNA and RNA
HMGB1	P09429 HMGB1	2.42	1.20 $\pm$ 0.04	1.12 $\pm$ 0.03	1.29 $\pm$ 0.09	Facilitating specific proteins DNA binding
hnRNP K	P61978 HNRPK	2.11	0.87 $\pm$ 0.05	0.85 $\pm$ 0.08	0.99 $\pm$ 0.03	pre-mRNA binding; poly(C) sequences binding and may be involved in the nuclear metabolism of hnRNAs
Protein S100-A4	P26447 S10A4	2	0.97 $\pm$ 0.01	0.95 $\pm$ 0.04	0.96 $\pm$ 0.01	Calcium-binding

Proliferating cell nuclear antigen (PCNA)	P12004 PCNA	2	0.83±0.04	0.74±0.07	0.88±0.01	Controlling of eukaryotic DNA replication
Metabolic enzymes						
Nucleoside diphosphate kinase A (NDPK-A)	P15531 NDKA	8	0.86±0.10	0.88±0.02	0.95±0.05	Tumor metastatic process associated Collagen binding, involved as a chaperone in biosynthesis of collagen
Serpin H1 precursor	P50454 SERPH	6.44	0.97±0.11	1.25±0.08	1.14±0.06	Redox regulation
Peroxiredoxin-1	Q06830 PRDX1	3.93	0.93±0.04	0.90±0.10	0.84±0.04	Induced by interferon, contains 10 TPR repeats
Interferon-induced protein with tetratricopeptide repeats 1 (IFIT-1)	P09914 IFIT1	2.59	0.94±0.05	0.80±0.11	0.82±0.01	Intracellular thiol proteinase inhibitor.
Cystatin-B (Stefin-B)	P04080 CYTB	2.01	1.10±0.11	1.15±0.05	1.13±0.07	
Structural proteins						
Filamin-A	P21333 FLNA	12	1.08±0.03	1.07±0.05	1.06±0.04	Branching of actin filaments and linking actin filaments to membrane glycoproteins
Lamin-A/C	P02545 LMNA	11.95	0.84±0.08	0.88±0.10	0.81±0.10	Components of the nuclear lamina Class-III intermediate
Vimentin	P08670 VIME	11.23	1.15±0.02	1.06±0.03	1.24±0.11	filaments found in various non-epithelial cells Connecting cell
Filamin-B	O75369 FLNB	8.47	1.05±0.03	1.07±0.05	1.12±0.02	membrane constituents to the actin cytoskeleton Anchoring the actin to
α -actinin-4	O43707 ACTN4	2.83	1.25±0.03	1.14±0.06	1.20±0.07	various intracellular structures
Microtubule-actin cross-linking factor 1, isoform 4 (MACF1)	Q96PK2 MACF4	2	1.61±0.05	1.53±0.20	1.27±0.11	Involved in cross-linking cytoskeletal proteins

Cell growth proteins

Ribonucleoside-diphosphate reductase large subunit (RRM1)	P23921 RIR1	6.01	1.10±0.03	1.11±0.04	1.13±0.20	Providing the precursors necessary for DNA synthesis
Proteasome subunit alpha type 3 (PSMA3)	P25788 PSA3	2	1.30±0.04	1.05±0.05	1.43±0.21	ATP-dependent multicatalytic proteinase
60S acidic ribosomal protein P1 (RPLP1)	P05386 RLA1	2	1.12±0.04	1.22±0.02	1.17±0.07	Playing an important role in the elongation step of protein synthesis

a) 115:114, the ratio of protein expression level in HepG2 transfected with HBx A (115) to HepG2 transfected with empty vector pXJ40 (114).

b) 116:114, the ratio of protein expression level in HepG2 transfected with HBx B (116) to HepG2 transfected with empty vector pXJ40 (114).

c) 117:114, the ratio of protein expression level in HepG2 transfected with HBx C (117) to HepG2 transfected with empty vector pXJ40 (114). (Data generated by Dr. Feng Huixing)

5.2.3 Validation of differentially expressed proteins by real-time RT-PCR

To confirm the differentially expressed proteins detected by LC-MS/MS, real-time RT-PCR was performed to quantify the expression of the selected genes at the mRNA level (Fig. 5.2B). Significant changes ($p < 0.05$) were analyzed based on three independent experiments for each transfection. In each one, the total mRNA were extracted and used as templates. Overall, the validation results were consistent with the LC-MS/MS data (Table 5.1, Fig. 5.2A). MACF1, Annexin A2 and PSMA3 showed up-regulation and S100A11, S100A4, and S100A6 showed down-regulation. However, hnRNP showed up-regulation in HBx genotype B while in genotypes A and C, it was down-regulated. Another inconsistency between real-time RT-PCR and LC-MS/MS data was on S100A11,

which was up-regulated in cells transfected with HBx genotype C, but down-regulated in all the three genotypes in LC-MS/MS result. The inconsistency may be due to the post-transcriptional regulation, post-translational modification and regulation.

Figure 5.2 Real-time RT-PCR analysis as validation of LC-MS/MS results. (A) Representative differential expression levels of proteins from the LC-MS/MS results. The four columns of each group represent samples from HepG2 cells transfected with empty pXJ40 vector (control), HBx of genotype A, B and C respectively. The normalized ratio between the HBx A, B, C transfected and control was shown. (B) Real-time RT-PCR validation of LC-MS/MS results. Three experiments were carried out

independently. The change folds were compared with the control (HepG2 cells transfected with empty vector pXJ40). The normalized expression levels were indicated by “asterisk”: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (Data generated by Dr. Feng Huixing)

5.2.4 Validation of differentially expressed proteins by Western blot

In addition to the mRNA level validation by RT-PCR, the changes of proteins were also confirmed by Western blot analysis.

As shown in Fig. 5.3 the last batch, β -actin served as loading control. Over-expressed HBx A/B/C was also analyzed by Western blot and the result showed even expression level. Based on the quantification of β -actin and HBx, the responding amounts of the samples were used for the analysis of the other proteins. Fig. 5.3 shows the results of S100A11, PSMA3 and Annexin A2. For S100A11, HepG2 cells transfected with pXJ40 shows the strongest expression and it was down-regulated by HBx A/B/C. In the case of PSMA3 and Annexin A2, the expressions of these two proteins were up-regulated by the HBx A/B/C. The trends of differentially changes of these proteins were consistent with the results of LC-MS/MS. Together with the results of real-time RT-PCR, the different protein expression in HepG2 cells was double confirmed by classical molecular biology experiments.

A

B

	pXJ40		HBx A		HBx B		HBx C	
Proteins	Adjusted density	Fold over pXJ40	Adjusted density	Fold over pXJ40	Adjusted density	Fold over pXJ40	Adjusted density	Fold over pXJ40
S100A11	2.705	1	2.262	0.836	1.760	0.651	1.445	0.534
PSMA3	0.509	1	0.910	1.788	0.737	1.448	0.885	1.737
Annexin 2	1.275	1	1.763	1.383	1.491	1.170	1.764	1.383

C

Proteins	pXJ40	HBx A	HBx B	HBx C
S100A11	1	0.94	0.86	0.86
PSMA3	1	1.30	1.05	1.43
Annexin 2	1	1.33	1.12	1.08

Figure 5.3 Western blot analysis of the proteins with differential expression in HepG2 cells transfected with HBx genotypes A/B/C. (A) Western blot analysis of the differential expressed proteins (Data generated by Dr. Feng Huixing). Expressions of HBx A/B/C were in comparative levels

among three genotypes; β -actin was internal control. (B) The density analysis of the western bands. The density of the band of each protein was adjusted by the internal control β -actin. Fold over the vector pXJ40 indicated that the density of pXJ40 was set as “1”, and the fold of others was shown respectively. (C) Summarization of the relative results from Table 5.1.

5.2.5 Comparison of HBV & HBx affected target proteins

Compared with the results from Chapter 4, sixteen unique proteins were identified in HBx-expressing HepG2 cells, which could be candidate targets of hypo- or hypermethylation by HBx. We found five common proteins that were down-regulated by both HBV (expressed in HepG2.2.15) and HBx (expressed in HepG2 transfected with HBx A-C) (Table 5.2).

Table 5.2 Common proteins identified affected by both HBV and HBx

Protein name	By HBV (HepG2.2.15)	By HBx (HepG2 transfected by HBx)
Annexin A2	Down-regulation	Up-regulation
Filamin A	Down-regulation	Up-regulation
Filamin B	Down-regulation	Up-regulation
Protein S100-A6	Down-regulation	Down-regulation
Protein S100-A4	Down-regulation	Down-regulation

5.3 Discussion

Among the five common proteins we identified to be affected by both HBV (expressed by HepG2.2.15) and HBx (expressed by transiently transfected HepG2), the two S100 proteins were consistently down-regulated. The S100 proteins belong to the calcium-binding EF-hand motif superfamily, and have a broad range of intracellular and extracellular functions [159]. In our results (described in Chapter 4), S100A6 was found to be down-regulated in HepG2.2.15 compared with HepG2, and this down-regulation was rescued by 5-aza-dC treatment. Here S100A6 was down-regulated in a percentage of 10-16% by HBx with different genotypes (Table 5.1). Such results are consistent with a recent report indicating that S100A6 is down-regulated by HBx due to hypermethylation in its promoter [114].

Apart from S100A6, S100A4 has been related with reduced expression of TSG such as p53 [166] and its over-expression in metastatic HCC cell lines compared with nonmetastatic liver cancer cell lines has implied that S100A4 is involved in HCC cell invasion and metastasis [19, 167]. Our results indicated that S100A4 was significantly down-regulated in HepG2.2.15 than HepG2 (Table 4.2); while in HBx-expressing HepG2, the down-regulation was not significant (Table 5.1, Fig. 5.2).

The down-regulation of S100A4 and S100A6 found in this study (Table 5.1) was in line with the results in Chapter 4. With the indication that we got from Chapter 4, the

down-regulation could be caused by DNA hypermethylation in their promoters by HBx.

Another S100 protein identified in this chapter is S100A11. S100A11 has been reported to have distinct roles depending on the tumor types, with a role of suppressing renal carcinoma [168] but promoting bladder carcinoma [169] and breast cancer [159]. For HCC, it is reported that S100A11 is over-expressed in metastatic HCC tissues compared with non-metastatic HCC [19], and it is involved in growth inhibition in HCC cells [170], indicating that S100A11 acts as a tumor suppressor in HCC. In our results, S100A11 was significantly down-regulated in HepG2 transfected with HBx of all genotypes, especially B and C (Table 5.1, Fig. 5.3), suggesting that HBx plays a role in inducing HCC development via suppression of S100A11 expression.

Apart from the group of signaling proteins including S100 proteins, NDPK-A (nucleoside diphosphate kinase A) was also found to be down-regulated in cells transfected with HBx, especially genotypes A and B compared with those not transfected (Table 5.1). NDPK-A was encoded by nm23-H1 gene, acting as a metastasis suppressor in certain human cancers such as neuroblastoma [115], melanoma and breast cancer [171], as well as HCC [172]. Similar as S100A11, this result suggested that HBx inhibited the expression of the tumor metastasis suppressor NDPK-A.

In addition to the individual discussion of above discussed proteins which are TSGs or acted as potential TSGs, it was noticeable that HBx had shown its role in down-regulating TSGs in a genotype-specific manner. The expression change folds of most proteins were

different in HBx genotypes A/B/C transfected HepG2 cells. Among the 24 proteins listed in Table 5.1, 11 proteins showed the most remarkable changes by HBx genotype B, 5 proteins by HBx genotype A and 8 proteins by HBx genotype C.

This genotype-specific manner may also help to explain another question. Although the results of quite a few proteins identified in this chapter were consistent with results from Chapter 4, some genes responded in the opposite direction, like Annexin A2 and FLN A&B, which were down-regulated by HBV in HepG2.2.15, they were up-regulated by HBx A-C. There could be two reasons. Firstly, it could be due to the genotype. Because in HepG2.2.15 we used in Chapter 4, the HBV integrated is genotype D; while in the study in Chapter 5, the genotypes we used of HBx were genotype A, B and C. Interestingly, variant outcome of genotype-specific HBV has also been inclined in clinical cases [51, 165]. Functionally, in cell migration assay performed by our colleague Dr. Feng Huixing, the HepG2 cells with HBx A showed stronger movement than genotypes B and C (unpublished data), which may provide some clue in HBV infection disease prognosis related to HCC. This genotype specific difference has also been reported in the interaction of HBx and Pin1 [35, 173], as well as the specific interaction between HBx genotype B and vinexin β [174]. Secondly, there might be different expression level between stable expressed HBV in HepG2.2.15 and forced over-expression of HBx in transiently transfected HepG2. And it's true that in nature, cells are initially infected with the entire HBV virus, not just HBx alone. However, there are clinical reports suggesting significant accumulation of viral RNAs containing HBV X but not C or S sequences in HCC patients [18, 175]. These suggest the frequent integration of the HBV genome, and selectively

over-expressed HBx in HBV-associated HCC patients, which indicating the expression of HBx alone may play an important role in hepatocarcinogenesis, and therefore worth further investigations.

In terms of the mechanism of genotype specificity, the functional differences may be caused by the sequence differences between genotypes. Variability in genetic changes of such relatively highly reserved protein has been observed previously [36], mainly located between amino acid 30 and 47 (Fig. 5.4). HBx of genotype B and C contained the most proline residues while genotype A contained more serines which may influence the binding affinity to SH3 domain containing proteins. This may make a structural and in turn functional difference among the viral HBx proteins of different genotypes which could lead to the variations in regulating target gene expression and disease onset.

Figure 5.4 Alignment of amino acids of HBx genotype A/B/C. HBx from HBV genome in pcDNA3.1 was cloned into pXJ40 and sequenced. In silico translation into amino acids was performed with BioEdit. Note that most of the HBx sequences are conserved with majority difference between 30-47 aa.

However, at the molecular level, little is known about genotype-specific effects of HBV/HBx on cellular processes. The detailed mechanism of genotype specific down-regulation of TSGs by HBx remains to be elucidated. Combined with our results in Chapter 4, such down-regulation could be caused via epigenetic regulations (such as DNA methylation) which might be further involved in HCC development. This indication hence leads us to the further mechanistic studies discussed in Chapter 6-7.

Chapter 6 HBx inhibits GSTP1 expression in hepatocellular carcinoma cells ^c

In addition to proteomic studies on proteins epigenetically suppressed or silenced by HBV as well as HBx, results shown in Chapter 6 and Chapter 7 focused on the epigenetic regulation of two TSGs (GSTP1 and DLEC1), regulated by HBx.

The reasons for selecting these two TSGs are as follows: first, the potential TSGs identified in Chapter 4 have also been frequently reported in proteomic studies with non-HBV background. Therefore, HBV might not be a specific factor involved in alterations in their expression; and further clinical validations should be required. On the other hand, not all the down-regulated proteins were identical in Chapter 4 and 5, suggesting that further analysis is needed to support their potential role as HBx regulated TSGs. In addition, GSTP1 and DLEC1 have been found to be hypermethylated in HCC in clinical studies [62, 117]. GSTP1 has also been found to be down-regulated in HBx-expressing HCC cell line in other proteomic studies [114]. DLEC1 has been found hypermethylated in HCC [62] with no link to HBV, but associated with hypoacetylation of histone in other cancers [140, 141]. Therefore, we selected GSTP1 and DLEC1 for further analysis instead of the proteins identified in our proteomic studies (Chapter 4 and 5).

^c Reproduced in part with permission from [Dandan Niu, Jianhua Zhang, et. al., Molecular Oncology, 3 (2009), 67-76] [2] Copyright [2009] @ Elsevier. Niu Dandan designed the experiments, performed most of the experiments, analyzed the data and wrote the paper. Mr. Zhang Jianhua helped with the ROS assay. Ms. Ren Yudan performed the operation of FACS machine and helped to analyze the data. Chen Wei Ning supervised the whole project and revised the manuscript.

Our results provide evidence suggesting that HBx plays a role in the epigenetic alterations in HBV/ HBx expressing HCC cell lines. Genotype-specific effects of HBx on the target genes were also discussed.

6.1 Introduction

Glutathione S-transferase P1 (GSTP1) belongs to the family of Phase II detoxification enzymes. Enzymes in this family catalyze the conjugation of various cytotoxic and carcinogenic influences, including aflatoxin B1, with glutathione. Among them, GSTP1 has been identified as a tumor suppressor gene with its high percentage of silenced expression in many types of human cancers. Promoter methylation of GSTP1 is the most studied mechanism for its silencing and it is an early event in many types of tumorigenesis, including prostate cancer, breast cancer, cholangiocarcinoma, as well as HCC, as reviewed by Tischoff *et al.* [56]. There are also reports showing that HBV positive patients have remarkably lower GST activity compared with HBV negative patients, which might indicate that cellular protection of human liver is compromised by HBV infection and further weakened during HCC development [117].

Mechanisms of activation of pathways involved in human cancer development have been identified by recent advances in epigenetics. Key players in epigenetic modifications include viral proteins such as HBx protein. The involvement of HBx in regional hypermethylation and global hypomethylation of target promoters has been studied [22]

and there is evidence indicating that HBx could activate a key enzyme during DNA methylation, DNA methyltransferase 1 (DNMT1), resulting in silence of some tumor suppressor genes and leading to the development of HCC [20, 21, 59]. In this study, we found that GSTP1 expression was completely depleted in HBV-integrated HepG2.2.15 cells due to the hypermethylation in its promoter region. Furthermore, we first identified and proved that it is HBx, especially HBx genotype D, that plays the key role in repressing the expression of GSTP1 and this repression was also confirmed by further functional studies like Reactive Oxygen Species (ROS) assay and apoptosis detection. Our results indicated that GSTP1 repression could be a mechanism of damaging cellular protection by HBV infection and replication. Our findings should facilitate the understanding of HBV and its influence on the epigenetic modulations causing tumorigenesis during HBV-associated HCC development.

6.2 Results

6.2.1 GSTP1 is significantly repressed in HepG2.2.15 compared with that in HepG2 and this repression could be rescued by 5-Aza-dC treatment

First we explored the GSTP1 expression pattern in HepG2 and HepG2.2.15 cells, and both of them treated by 5-Aza-dC. As shown in Fig. 6.1, both real-time RT-PCR and Western blot give respectively the evidences on mRNA level (Fig. 6.1A) and protein level (Fig. 6.1B) that, GSTP1 was normally expressed in HepG2 (column 1 in Fig. 6.1A and lane 1 in Fig. 6.1B) but completely depleted in HepG2.2.15 (column 3 in Fig. 6.1A and

lane 3 in Fig. 6.1B), which steadily expressed HBV genome. Further, after the treatment of DNMT1 inhibitor 5-Aza-dC, GSTP1 expression in HepG2 was slightly elevated (column 2 in Fig. 6.1A) while significantly rescued in HepG2.2.15 (column 4 in Fig. 6.1A and lane 4 in Fig. 6.1B).

5-Aza-dC is expected to exert its demethylation function by sequestering DNMT1 and decreasing the cellular concentration of DNMT1, leading to genomic DNA demethylation in the course of successive cell divisions [86, 90]. Our results indicated that GSTP1 expression could be inhibited by HBV genome expression and this alteration might occur due to the DNA hypermethylation in GSTP1 promoter. Our results also agree with some clinical studies indicating that GSTP1 activity was lower in HBV positive patients than those who were negative [117], and the frequency of GSTP1 promoter hypermethylation was significantly higher in HCC than corresponding non-tumor tissues from HBsAg-positive patients, but not HBsAg-negative controls [112].

Figure 6.1 GSTP1 expression in HepG2 and HepG2.2.15 with or without treatment of 5-Aza-dC. (A) Real-time RT-PCR showing the expression of GSTP1 on mRNA level in HepG2 and HepG2.2.15, and both of them treated by 2.5 μ M 5-Aza-dC for 72 hr. Three independent experiments were carried out. Compared with HepG2 untreated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (B) Western blot

showing GSTP1 expression on protein level (up panel), with the bottom panel of β -actin as loading control.

6.2.2 Promoter region of GSTP1 in HepG2.2.15 is completely methylated and partially demethylated after 5-Aza-dC treatment

To further confirm whether the GSTP1 expression alteration was caused by DNA methylation or not, we performed MSP to test the promoter DNA methylation status of GSTP1 in the cells. Both of HepG2 and HepG2.2.15 cells were analyzed after the treatment of 5-Aza-dC or mock treatment, as described in methods part 3.1.2.4. It is shown in Fig. 6.2 that GSTP1 in HepG2 cells is partially methylated (both bands in M and U lanes, Lane 1 and 2), while in HepG2.2.15 it is completely methylated (only band in M lane but not in U lane, Lane 5 and 6). This result agreed with the GSTP1 expression pattern in both of the cells (Fig. 6.1), and these combined to suggest that GSTP1 could be expressed in HepG2 when some of the alleles were unmethylated, but were totally silenced in HepG2.2.15 due to all the methylated alleles.

After 5-Aza-dC treatment, parts of the methylated alleles were demethylated and thus the percentage of PCR products in U lane of HepG2 was slightly increased while products in U lane of HepG2.2.15 clearly appeared. This result provided further evidence that GSTP1 expression inhibition in HepG2.2.15 compared with HepG2 was caused by GSTP1 promoter DNA hypermethylation.

Figure 6.2 MSP analysis of the methylation status of the GSTP1 promoter in HepG2 and HepG2.2.15 cells. The presence of PCR products in Lane M indicates the presence of methylated alleles of GSTP1 while the presence of PCR products in Lane U indicates the presence of unmethylated alleles of GSTP1. Water was used as the negative control.

6.2.3 GSTP1 expression is significantly repressed in HBx D transiently transfected HepG2 cells

To study the potential factors from HBV integration in HepG2.2.15, causing such GSTP1 expression alteration, we focused our attention on one of the four proteins encoded by HBV genome, HBx, well-known as a versatile regulatory factor and indicated to be involved in epigenetic regulation. We wanted to see whether HBx plays a role in this process.

We constructed plasmids containing HBx coding sequences with different genotypes, genotype A, B, C and D, transfected them as well as empty vector pXJ40 into HepG2 cells, and tested GSTP1 expression by Western blot assay. As shown in Fig. 6.3, when HBx protein was expressed evenly, there was suppression of GSTP1 expression by HBx, especially by HBx genotype D.

Figure 6.3 Western blot analysis of GSTP1 expression in HBx genotype A-D transiently transfected HepG2 cells. In the upper Western results, the first panel was blotted with anti-GSTP1 antibody, while second panel was blotted with anti-HA antibody to show the HBx protein expression as well. β -actin was included as the loading control. pXJ40 transfected HepG2 served as the blank vector control. The table in the bottom showed the adjusted density of GSTP1 according to internal control β -actin, and the fold of expression in each HBx genotype transfected cells compared with the empty vector pXJ40.

6.2.4 ROS levels increased in HBV-expressing HepG2.2.15 and HBx transfected HepG2 cells

According to the traditional understanding of GST enzymes, the major function of GSTP1 should be detoxification of electrophiles or oxidant carcinogens by glutathione conjugation. There are a wide variety of electrophilic substrates, including both

endogenous [e.g. by-products of reactive oxygen species (ROS) activity] and exogenous (e.g. polycyclic aromatic hydrocarbons) [176]. In order to confirm the inhibition effect of GSTP1 by HBx D, we further detected the cellular ROS level using an ROS detection kit. With this kit, ROS level could be indicated by the number of cells with green fluorescence: the more fluorescence these cells display, the higher the ROS level is. As shown in Fig. 6.4A, ROS level in HepG2.2.15 (Fig. 6.4A, b) was significantly higher than that in HepG2 (Fig. 6.4A, e); and could be reduced by 5-Aza-dC treatment in both of the cell lines (Fig. 6.4A, HepG2.2.15: c compared with b; HepG2: f compared with e).

In Fig. 6.4B, ROS levels in HepG2 cells transiently transfected with HBx A-D were all detected. Compared with cells transfected by empty vector pXJ40, certain increase of ROS level could be seen in cells transfected by all the genotypes of HBx, most significantly by HBx. That is to say, HBx D could increase the ROS level, the same effect as that in HepG2.2.15, which has HBV integrated. The ROS results further confirmed the link between HBx genotype D and the repression of GSTP1 expression.

A

	Fluorescent view	Light view	Percentage of positive cells
a HepG2.2.15 TBHP treated (ROS assay positive)			86.11%
b HepG2.2.15 mock treated			79.07%
c HepG2.2.15 5-Aza-dC treated			45.83%

	Fluorescent view	Light view	
d HepG2 TBHP treated (ROS assay positive control)			76.92%
e HepG2 Mock treated			3.54%
f HepG2 5-Aza-dC treated			1.25%

Figure 6.4 ROS assay detecting the ROS levels in HepG2.2.15 and HBx transiently transfected HepG2 cells. (A) ROS levels in HepG2 and HepG2.2.15 cell lines. Fluorescent view (left panel) was presented, as well as the light view (right panel) serving as cell density control. a) HepG2.2.15 treated by TBHP (tert-butyl hydroperoxide) which serves as the assay positive control for the successful induction of ROS; b) HepG2.2.15 mock treated by only DMSO in the same volume as that of 5-Aza-dC in c); c) HepG2.2.15 treated by 5-Aza-dC; d) HepG2 treated by TBHP as the assay positive control; e) HepG2 mock treated by only DMSO; f) HepG2 treated by 5-Aza-dC. (B) ROS levels in HepG2 cells transfected by HBx A-D. HepG2 cells transfected by the blank vector pXJ40 was also included as the negative control. The numbers of the ROS positive cells and the total number of cells were counted and the percentage of the positive cells was listed on the right-hand side of each panel.

6.2.5 HBx genotype D increases apoptosis of HepG2 cells

As well known, ROS are involved in several different cellular processes ranging from apoptosis and necrosis to cell proliferation and carcinogenesis. In our previous research, we found that cellular apoptosis could be induced by replication of HBV genomes [67], so here with the above identification that HBx could increase cellular ROS level, we further explored whether the ROS level increased by HBx could also lead to apoptosis.

Therefore, apart from HepG2.2.15 cells, we transfected HepG2 cells with pXJ40-HBx A-D, as well as the empty vector pXJ40, labeled them with the Annexin-V-FLUOS Staining Kit and analyzed the apoptosis of cells by flow cytometry. As shown in Fig. 6.5A, more than 95% of the untreated HepG2 cells (negative control) were normal living cells and only 2.6% were apoptotic. While in Fig. 6.5B, HepG2 cells treated with 50 μ M cisplatin for 16 hr were involved as the positive control of induced apoptosis, and the apoptotic cells reached to 73.6%. Compared with HepG2 cells transfected with empty vector pXJ40, which had 18.01% as the apoptotic cells, except HBx C transfected HepG2, either HepG2.2.15 (35.8%) or HepG2 cells transfected with HBx A (24.3%), B (27.68%) and D (51.58%) displayed a higher percentage of apoptotic cells, especially HBx genotype D.

These results suggested that either HepG2.2.15 with HBV integrated, or HepG2 transfected by HBx genotype D could significantly increase the cell apoptosis.

Figure 6.5 Flow cytometry analysis of apoptosis in HepG2.2.15 and HBx transfected HepG2 cells.

(A) HepG2 cells without treatment served as negative control and were used to get the analysis settings. (B) HepG2 cells treated with Cisplatin were used as the apoptosis positive control. (C) HepG2.2.15 cells. (D)-(H) HepG2 cells transfected with either pXJ40 vector, or pXJ40-HBx A/D. FL1-H indicates the Annexin staining result while FL2-H indicates the PI staining result. Therefore, LL (lower left) gives the normal (living) cells with low Annexin and low PI staining and LR (lower right) gives the apoptotic cells with high Annexin and low PI staining, while UR (upper right) shows the necrotic cells with high Annexin and high PI staining. Experiments were carried out three times with one typical set of results shown.

6.3 Discussion

GSTP1, which is one of the detoxification enzymes defending cells against damage mediated by electrophilic or oxidant carcinogens, has been considered as a tumor suppressor gene which is usually repressed or even silenced in several types of human cancers. Some of the clinical researches have suggested that the hypermethylation on the promoter CpG sites of GSTP1 has relationship with HBV infection, especially in HBV-associated HCC [61, 112, 117]. However, the molecular processes underlying such phenotype and the detailed contribution of HBV are poorly understood. In this study, we explored the role of HBx protein, especially HBx genotype D, in the GSTP1 hypermethylation and silenced expression by HBV infection.

To study this problem, we employed two cell lines HepG2 and HepG2.2.15. HBV genome expression in HepG2.2.15 cells has been validated as shown in Fig. 4.1, which suggested that the HepG2.2.15 cell line we were using could express HBV genome as expected.

We first explored our target protein GSTP1 expression on either mRNA level (Real-time RT-PCR, Fig. 6.1A) or protein level (Western blotting, Fig. 6.1B) in HepG2 as well as HepG2.2.15 cells. It was clear that, GSTP1 expression could be detected in HepG2 (Fig. 6.1A Column 1 and Fig. 6.1B Lane 1) but was completely depleted in HepG2.2.15 (Fig. 6.1A Column 3 and Fig. 6.1B Lane 3). This result was consistent with

those previously mentioned clinical reports [61, 112, 117], which indicated that GSTP1 was silenced in HBV-associated HCC. But as to the GSTP1 expression in HepG2, our result disagreed with one of the published studies [61], which showed that there was no GSTP1 expression detectable in HepG2 because its promoter in HepG2 was totally methylated. This discrepancy might due to different culture conditions or different passage times in each experiment. Furthermore, the GSTP1 expression level in both of the cells could be increased (from none to detectable in the case of HepG2.2.15) after the treatment of demethylation reagent 5-Aza-dC. The increase in HepG2 was not as significant as that in HepG2.2.15; this may due to that the efficiency of 5-Aza-dC treatment was not high enough, but the trend was still there. Considering some clinical reports indicating that the frequency of GSTP1 promoter hypermethylation was significantly higher in HCC than corresponding non-tumor tissues from HBsAg-positive patients, but not HBsAg-negative controls [112], our results probably suggested that DNA methylation might play a role in GSTP1 repression in HepG2.2.15 cells.

Therefore, to further check the potential DNA methylation mechanism for depletion of GSTP1 expression in HepG2.2.15, MSP was carried out to check DNA methylation status of GSTP1 promoter in both HepG2 and HepG2.2.15 cells. It is shown in Fig. 6.2 that, while there were both methylated and unmethylated alleles of GSTP1 in HepG2, all the alleles of GSTP1 in HepG2.2.15 were methylated. This result consisted well with the GSTP1 expression patterns in both of the cell lines (Fig. 6.1). That was, HepG2 could express GSTP1 due to its partially unmethylated GSTP1 promoter, while HepG2.2.15 was not able to express the gene because all of the GSTP1 alleles were methylated. In addition,

as expected, 5-Aza-dC treatment was able to change some of the GSTP1 alleles in HepG2.2.15 from methylated (Fig. 6.2 Lane 5-6) into unmethylated (Fig. 6.2 Lane 7-8); although the change of the methylation pattern in HepG2 cells after 5-Aza-dC treatment was not that significant, we still can see lower density of methylated band (Fig. 6.2 Lane 3 compared with Lane 1) and higher density of unmethylated band (Fig. 6.2 Lane 4 compared with Lane 2). The MSP results provided evidence directly to confirm the role of DNA hypermethylation in GSTP1 repression in HepG2.2.15 by HBV.

With the above evidence, we proposed that HBV presented in HepG2.2.15 caused hypermethylation of GSTP1 promoter region and thus totally inhibited its expression. The next question was what factor or factors would be in charge of this change? As is well known, one of the four proteins encoded by HBV genome, HBx is considered as a quite versatile viral protein, involved in many cellular functions such as transcriptional regulation, DNA repair, cell adhesion and apoptosis. What's more, the functions of HBx have been expanded to the field of epigenetics. It is reported that HBx can play a role in regulating DNA methylation, via regulating the expression of DNMT 1 [20, 21]. For instance, some studies have showed that the HBx protein could repress E-cadherin [21, 59] and p16^{INK4a} [20] expression via activation of DNMT1. So here it is reasonable to explore whether HBx also plays a similar role in GSTP1 repression.

To achieve this, we constructed plasmids containing HBx with different genotypes, from HBx genotype A to genotype D, and then transfected them into HepG2 cells to see whether the forced expression of HBx has something to do with GSTP1 expression.

Western blot results in Fig. 6.3 indicated that GSTP1 expression could be significantly inhibited by HBx genotype D; this primarily confirmed our hypothesis that HBx did have an effect on GSTP1 expression.

With GSTP1 expression down-regulated, the intracellular microenvironment generating ROS may be altered accordingly. Two functional assays, ROS detection and apoptosis analysis were performed subsequently and provided further evidence that suppression of GSTP1 expression by HBx D transfected HepG2 as well as HBV integration in HepG2.2.15, might lead to ROS increase (Fig. 6.4) and apoptosis (Fig. 6.5). However, it seems somewhat incompatible with the concept that HBV infection should favor the hepatocarcinogenesis, which involves decreased rather than increased apoptosis. Actually, there are contradictory data about the HBx effect on cellular apoptosis, either anti-apoptosis [177] or pro-apoptosis [178-180], which might be due to differences in cells and experimental systems. As reviewed by Lohong Ye, etc al., HBx can either inhibit or promote cell apoptosis in a dose-dependent manner. When HBx is expressed at high levels, it promotes apoptosis; whereas it inhibits apoptosis when expressed at physiological levels [181]. In our study, HBx was over-expressed in cells and increased apoptosis. Therefore, it did not conflict but support the functional significance of the altered GSTP1 expression by HBx.

In terms of the role of GSTP1, it also seems contradictory that GSTP1 as a TSG, the down-regulated GSTP1 can induce, not inhibit apoptosis, which seems the opposite side of tumorigenesis. Actually, as reviewed by Scott W.Lowe et.al, although the apoptosis and

apoptosis-related genes have a profound effect on the malignant phenotype, compelling evidence indicates that other oncogenic changes promote apoptosis [182]. For example, excessive exposure to the skin of UV radiation induces apoptosis, which presumably serves to eliminate heavily damaged cells. UV radiation induces apoptosis, and loss of function of p53 leads to the survival of these damaged cells thereby initiating tumor development [183]. Therefore although the detailed signal pathway or mechanism remains unknown, our result that down-regulated GSTP1 expression leads to increased apoptosis, does not conflict with the role of GSTP1 as a tumor suppressor. On the other hand, besides our results, another report also suggested that GSTP1 reduces cellular proliferation and protects against apoptosis [176]. Furthermore, our results agree with several clinical studies that GSTP1 expression were significantly down-regulated due to hypermethylation in HBV-associated HCC [112, 117], and provide further evidence at the molecular level to suggest that the cellular protection within human liver is compromised by HBV infection, especially HBx function, and further decreased during hepatocellular tumorigenesis. Regarding the genotype-specific function of HBx D, our results provides further clues at molecular level for the clinical observation that, HBV D is associated with more severe liver disease and may predict the occurrence of HCC in patients [184].

Conclusively, we found that GSTP1 expression was completely depleted in HBV integrated HepG2.2.15 cells due to the hypermethylation in its promoter region, and it was HBx, especially HBx genotype D, that played the key role in repressing GSTP1 expression. This repression was also confirmed by further functional studies such as ROS assay and apoptosis detection. These findings should facilitate the understanding of HBV

and its influence on the epigenetic modulations contributing to HBV-associated HCC development.

Chapter 7 HBx regulates DLEC1 expression via DNA demethylation and histone acetylation in HCC cells

7.1 Introduction

Extensive studies on HCC development have focused on genetic abnormalities, including chromosomal deletions and rearrangements, aneuploidy, gene amplifications and mutations in TSGs [185, 186]. Recently an epigenetic model of the HCC development has also been established [22]. Apart from DNA methylation we discussed in the previous chapters, alteration of histone acetylation can also regulate gene expression. Histone acetylation is one form of the posttranslational histone modifications, which have been implicated in epigenetic processes [81]. Histone acetylation and DNA methylation at CpG sites are both involved in the way that DNA is packaged and can therefore regulate gene expression at the transcriptional level [187, 188]. It is generally believed that acetylation of lysine residues at the N-terminus of histone proteins results in the removal of positive charges, hence reducing the affinity between histone and DNA. This allows RNA polymerase and transcription factors easier access to the promoter region [187]. As a result, histone acetylation enhances transcription while histone deacetylation represses transcription. Histone acetylation is catalyzed by histone acetyltransferases (HATs) and histone deacetylation is catalyzed by histone deacetylases

(HDACs) [189].

DLEC1 (Deleted in Lung and Esophageal Cancer 1), as a functional tumor suppressor gene, has been found to be frequently silenced in human cancers, including non-small cell lung cancer [47], esophageal cancer [140], colon and gastric cancers [127, 190], nasopharyngeal cancer [191], epithelial ovarian cancer, as well as HCC [62]. Unlike GSTP1 analyzed in Chapter 6, which is only modulated by DNA hypermethylation, the silence of DLEC1 is subject to both DNA hypermethylation and histone hypoacetylation [140, 141]. It was therefore selected to evaluate further the impact of HBx in HBV-mediated epigenetic modulations.

In the case of HCC, key players in epigenetic modifications include viral proteins such as HBx. The involvement of HBx in regional hypermethylation and genome-wide hypomethylation of target promoters, results in silence of tumor suppressor genes and/or activation of oncogenes leading to development of HCC [22]. Recent evidence has revealed an association of HBV genotypes, characterized by their distinct geographic distribution, with distinct clinic manifestations. For example, genotype B appears to be more inclined to develop HCC, whereas genotype A and C cause more hepatitis and cirrhosis [192]. However, the understanding of the genotype specificity of HBV, including epigenetic regulations of TSGs by HBx, remains to be elucidated. In the present chapter, the effects of HBx on regulating the TSG DLEC1 through epigenetic mechanisms (including DNA methylation and histone acetylation) were analyzed.

7.2 Results

7.2.1 Epigenetic alterations of DLEC1 in HCC cell lines

7.2.1.1 Promoter of DLEC1 was hypermethylated in HepG2 and Huh7

To assess promoter DNA methylation status, we performed bisulfite genome sequencing (BGS) for DLEC1 promoter in two HCC cell lines, HepG2 and Huh7. The promoters from both of the cells were analyzed after the treatment of 5-Aza-dC or mock treatment. Results shown in Fig. 7.1A showed that both HepG2 and Huh7 were hypermethylated in their promoters. The percentage of methylation was found to be 89.47% in HepG2 (Fig. 7.1A row 1) and 84.21% in Huh7 (Fig. 7.1A row 3). On the other hand, after the treatment of DNA methyltransferase inhibitor 5-Aza-dC, the methylation percentage decreased to 69.74% of HepG2 (Fig. 7.1A row 2) while 29.34% of Huh7 (Fig. 7.1A row 4). The HepG2 result was consistent with the previously reported data [62]. Our results indicated that DLEC1 was significantly hypermethylated in both HCC cell lines. A representative sequencing data of 3 CpG sites in DLEC1 promoter shown in Fig. 7.1B indicated the different patterns of the CpG methylation status with or without 5-Aza-dC treatment in both of the cell lines. Specifically, three CpG sites of HepG2 were all methylated (Fig. 7.1B, upper left panel) while after 5-Aza-dC treatment (Fig. 7.1B, upper right panel), one of them (rectangle in left panel with arrow) became unmethylated

(ellipse in right panel with arrow). In the lower row, two CpG sites of Huh7 were methylated (Fig. 7.1B, lower left panel), and after 5-Aza-dC treatment, all of the three were demethylated (lower right panel).

To correlate the DNA hypermethylation of DLEC1 with its expression, RT-PCR was performed to detect its expression in both HepG2 and Huh7 treated with 5-Aza-dC. As shown in Fig. 7.1C, with the internal control of β -actin was even in each treatment, DLEC1 expression in both HepG2 and Huh7 after 5-Aza-dC treatment was up-regulated compared with those untreated cells. This confirmed that DLEC1 expression was partially inhibited in the two HCC cell lines by DNA hypermethylation.

A

Figure 7.1 DLEC1 promoter hypermethylation and its expression in HCC cell lines. (A) Promoter methylation analysis by bisulfite genome sequencing of DLEC1 5' CpG sites in both of HepG2 and Huh7 with or without treatment of 5-Aza-dC. 19 CpG dinucleotides were analyzed in the 5' promoter of DLEC1 (represented as 19 columns). 6-8 clones of each sample were sequenced and the results were presented as follow: Open area indicates the percentage of unmethylated CpG sites, while dark area indicates percentage of methylated CpG sites. The quantitative result in percentage of methylated CpG sites was listed on the right of the column groups. Three independent experiments were carried out and representative result from one of them was listed here. (B) Representative bisulfite sequencing results of the DLEC1 promoter region showing the CpG methylation status of bisulfite treated DNA from two cell lines mock treated or 5-Aza-dC treated. Rectangles indicate the methylated CpG sites, while ellipses for unmethylated CpG sites. Arrows mark the CpG sites whose methylation status was changed by 5-Aza-dC treatment. (C) RT-PCR analysis of DLEC1 expression in two cell lines mock treated or 5-Aza-dC treated.

7.2.1.2 Promoter of DLEC1 showed histone hypoacetylation in HepG2 and Huh7

Apart from the DNA hypermethylation, we also analyzed the level of histone acetylation of DLEC1 in the two cell lines by chromatin immunoprecipitation (ChIP)

assay. For each cell line, according to the manual provided with the kit, 1% of the DNA prior of immunoprecipitation for each treatment was used as input control. As shown in Fig. 7.2A, both HepG2 (Fig. 7.2A, upper panel) and Huh7 (Fig. 7.2A, lower panel) had low level of histone H4 acetylation (HepG2 lane 5, Huh7 lane 7), and undetectable result of histone H3 acetylation (HepG2 lane 3, Huh7 lane 4). After the treatment of histone deacetylase inhibitor TSA, the levels of both acetylated histone H3 and H4 were significantly increased in both cell lines (HepG2 lane 4 & 6, Huh7 lane 5-6 & 8-9). In HepG2 cells, the band for acetylated histone H3 was detected (HepG2 lane 4) while level of acetylated histone H4 was greatly up-regulated (HepG2 lane 6). Similar results were obtained for Huh7 (Huh7 lane 5-6, lane 8-9).

To correlate the expression of DLEC1 with histone acetylation status, RT-PCR was performed for the two cell lines. The RT-PCR results indicated that the expression of DLEC1 was obviously reactivated in both of the cells after TSA treatment (Fig. 7.2B).

Figure 7.2 Histone H3 & H4 hypoacetylation in DLEC1 5' region and its expression in HCC cell lines. (A) ChIP analysis of 5' region of DLEC1 in HepG2 and Huh7 mock treated or TSA treated. DNA

sequences cross-linked to acetylated histone H3 and H4 were co-immunoprecipitated as part of the chromatin complex with the help of antibodies specific to acetylated histone H3 and H4. The reaction without antibody immunoprecipitation (no Ab) serves as negative control. Input indicated 1% of total DNA prior to immunoprecipitation. (B) RT-PCR analysis of DLEC1 expression in two cell lines mock treated or TSA treated.

7.2.2 DLEC1 was not reactivated in HBV-producing HepG2.2.15 cells

To explore the role of HBV in the epigenetic regulations of DLEC1 in HCC cells, we analyzed both DNA methylation and histone acetylation of DLEC1 in the HBV-producing HepG2.2.15 cells. The DNA methylation, histone H3 & H4 acetylation levels, as well as mRNA expression of DLEC1 were shown in Fig. 7.3. The result of methylation analysis by BGS was shown in Fig. 7.3A. The methylation percentage of DLEC1 in HepG2.2.15 (46.49%, Fig. 7.3A) was significantly lower than that in HepG2 (89.47%, Fig. 7.1A). It was further decreased to 33.06% after the treatment with 5-Aza-dC (Fig. 7.3A). For histone acetylation, ChIP result indicated that both acetylation levels of histone H3 and H4 in HepG2.2.15 (Fig. 7.3B, lane 3 & 5) were increased compared with those in HepG2 (Fig. 7.2A, HepG2 lane 3 & 5). In addition, in HepG2.2.15 the treatment with TSA resulted in an increase of the histone H3 acetylation (Fig. 7.3B, lane 4). The increase of histone H4 acetylation (Fig. 7.3B, lane 6) was not as significant as H3, which might due to the fact that, the level of acetylated histone H4 without TSA (Fig. 7.3B, lane 5) treatment was already high.

In contrast to HepG2 and Huh7 cells, the expression of DLEC1 in HepG2.2.15 was not detected by RT-PCR, neither in the cells without treatment nor treated with 5-Aza-dC / TSA (Fig. 7.3C).

Figure 7.3 DNA methylation and histone acetylation status of DLEC1, and its expression in HepG2.2.15. (A) Promoter methylation analysis by bisulfite genome sequencing of DLEC1 5' CpG sites in HepG2.2.15 with or without treatment of 5-Aza-dC. Open area indicates the percentage of unmethylated CpG sites, while dark area indicates percentage of methylated CpG sites. The quantitative result in percentage of methylated CpG sites was listed on the right of the column groups. Three independent experiments were carried out and representative result from one of them was listed here. (B) ChIP analysis of 5' region of DLEC1 in HepG2.2.15 mock treated or TSA treated. Input indicated 1% of total DNA prior to immunoprecipitation. (C) RT-PCR analysis of DLEC1 expression in HepG2.2.15 treated by either 5-Aza-dC or TSA.

7.2.3 DLEC1 expression was up-regulated in HBV replicating HepG2 cells

Apart from the HepG2.2.15 which contains HBV genotype D, we also evaluated DLEC1 expression in HepG2 cells transfected with HBV genotypes A, B and C. A set of HBV genome-carrying expression plasmids was constructed, with the HBV samples from Singapore clinical samples, and transfected into HepG2 cells. To analyze the variation of expression level more precisely, we carried out real-time RT-PCR to compare the expression of DLEC1 in HepG2 transfected by HBV (Fig. 7.4).

As shown in Fig. 7.4, there are genotype specific modulations of DLEC1 expression by HBV genomes. HepG2 cells transfected by HBV genotype A (Fig. 7.4 column 2) showed a 40% increase in DLEC1 expression compared to cells transfected by empty vector pcDNA3.1 (column 1). HBV genotype B had about 20% repression (column 3) of DLEC1 expression. These results indicated that, different HBV genotypes had various effects on DLEC1 expression.

Figure 7.4 Real-time RT-PCR analysis of DLEC1 expression in HBV genotype A-C expressing HepG2. pcDNA3.1 was included as empty vector control. Experiment was done by three times.

Experimental differences were tested for statistical significance using t-test. * $p < 0.05$; ** $p < 0.01$.

7.2.4 HBx genotype A played a role in up-regulation of DLEC1

To understand the underlying mechanisms of the above phenomena, HBV X protein (HBx) was picked out as a potential factor in the epigenetic modulations. Some of the evidence suggest genotype specific effects of HBx in host cells [2, 36].

Therefore, we transfected plasmids containing HBx coding sequences with different genotypes (A, B, C and D) into HepG2 and Huh7 cells, as well as the empty vector pXJ40. HBx of various genotypes was confirmed to be normally expressed by Western blotting assay with HA antibody (Fig. 7.5A). DLEC1 expression was examined by real-time RT-PCR (Fig. 7.5B & C). As shown in Fig. 7.5B, in HepG2, HBx with different genotypes had different effects on DLEC1 expression compared with the vector pXJ40. Specifically, DLEC1 expression was significantly increased by HBx genotype A (Fig. 7.5B, column 2) and inhibited by HBx B & D (Fig. 7.5B, column 3 & 5). The effect of HBx C on expression of DLEC1 was not significant (Fig. 7.5B, column 4). In Huh7 cells, results with similar pattern were obtained (Fig. 7.5C). Results from both of the cell lines suggested that there was an HBx genotype specific alteration of DLEC1 expression in these cells.

Figure 7.5 DLEC1 expression in HBx genotype A-D expressing HepG2 and Huh7. (A) Western analysis of HBx expression in transfected Huh7. HBx expression was detected by HA tag contained in the vector pXj40, and β -actin was also detected as the internal control. (B) Analysis of DLEC1 expression in HBx genotype A-D expressing HepG2 by real-time RT-PCR. (C) Analysis of DLEC1 expression in HBx genotype A-D expressing Huh7 by real-time RT-PCR. Experiment was done three times. pXJ40 was included as an empty vector control. Experimental differences were tested for

statistical significance using t-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

7.2.5 The epigenetic regulations of DLEC1 in HBx transfected Huh7 cells

Inspired by the genotype specific effects of HBx on DLEC1 expression, correlation of such effects to epigenetic alterations was examined. Both BGS and ChIP assay were performed to evaluate the DNA methylation and histone acetylation status of Huh7 transfected by HBx of different genotypes (Fig. 7.6).

Results shown in Fig. 7.6A indicated that the percentage of DNA methylation of DLEC1 in Huh7 transfected by HBx A-D varied from each other. The lowest percentage of methylation was in Huh7 transfected by HBx A (40.79%), which was half of that in cells transfected by vector pXj40 (85.53%). HBx B (63.16%) and C (69.86%) also led to lower percentage of methylation, but HBx D (84.93%) had no obvious effect on DNA methylation compared with the vector.

On the other hand, histone acetylation status was explored by ChIP indicated in Fig. 7.6B. 1% of the total DNA before the immunoprecipitation was used as the input control. Compared to the input control, the acetylated histone H4 (Fig. 7.6B, panel 2) was increased in Huh7 transfected by HBx, especially in HBx genotype A (Fig. 7.6B, panel 2 lane 2), while for acetylated histone H3, there were no significant changes in different cells (Fig. 7.6B, panel 3). Combined with DNA methylation alterations, we believe that HBx, especially HBx genotype A, induces epigenetic alterations (DNA demethylation and

increased histone acetylation) and therefore up-regulates the expression of DLEC1.

Figure 7.6 DNA methylation and histone acetylation status of DLEC1 in Huh7 expressing HBx genotypes A-D. pXJ40 was included as an empty vector control. (A) Promoter methylation analysis by bisulfite genome sequencing of DLEC1 5' CpG sites in Huh7 transfected by HBx genotypes A-D. Open area indicates the percentage of unmethylated CpG sites, while dark area indicates percentage of methylated CpG sites. The quantitative result in percentage of methylated CpG sites was listed on the right of the column groups. Three independent experiments were carried out and representative result from one of them was listed here as the upper panel; the lower panel summarized the results from all three experiments. (B) ChIP analysis of 5' region of DLEC1 in Huh7 expressing HBx. Input indicated 1% of total DNA prior to immunoprecipitation. Experiments were done three times. Experimental differences were tested for statistical significance using *t*-test. ***p* < 0.01.

7.3 Discussion

DLEC1 has been identified to be frequently silenced in human cancers including HCC, while DLEC1 over-expression in HCC cell lines led to a decrease in colony formation, cell growth and cell size, and an induction of G1 arrest in cell cycle [62]. The silencing of DLEC1 is considered to be associated with DNA hypermethylation [47, 62], as well as histone hypoacetylation [140, 141, 189]. However, the present epigenetic studies of DLEC1 are all about the identification and confirmation of DLEC1 silencing due to epigenetic alterations in various human cancers; the pathogenic factors which could be involved are still unclear.

In the case of HCC, key players in epigenetic modifications include viral proteins, such as HBx. As a versatile viral protein, HBx has been identified to have many functions, such as transcriptional co-activator, cellular proteasome inhibitor, and oncoprotein [193]. It is involved in many signal pathways, which makes it not only essential for virus infection and integration into host genome, but also quite active in hepatocellular carcinogenesis. However, the involvement of HBx in epigenetic modifications, especially DNA methylation of genes of host cells has received attention only quite recently [20-22]. Recent studies revealed that HBx can activate DNMTs and then repress certain down-stream tumor suppressor genes. In this chapter, we focused on the DLEC1 expression in HCC cell lines, and explored the role of HBV, especially its X protein (HBx) in the regulation of DLEC1 expression.

We first detected the DLEC1 expression, as well as DNA methylation and histone acetylation status of DLEC1 in two HCC cell lines, HepG2 and Huh7. The DNA methylation of DLEC1 promoter was highly methylated in both of the cells, HepG2 89.47% and Huh7 84.21% (Fig. 7.1A, panel 1 & 3), and was significantly demethylated by DNA methyltransferase inhibitor 5-Aza-dC: HepG2 69.74% while Huh7 29.34% (Fig. 7.1A, panel 2 & 4). DLEC1 expression was increased in both cell lines after 5-Aza-dC treatment (Fig. 7.1C), which could be correlated with their methylation status (Fig. 7.1A). The result of HepG2 was also supported by another report [62]. Combined with our results, it indicated that DLEC1 was suppressed in HepG2 and Huh7 and the DNA hypermethylation could play a role in this suppression.

On the other hand, promoter region of DLEC1 showed histone H3 & H4 hypoacetylation in the two cell lines, and their acetylation levels were greatly increased after treatment with deacetylase inhibitor TSA (Fig. 7.2A). Further, DLEC1 expression was found to be significantly correlated with their histone acetylation status (Fig. 7.2B). Interestingly, compared with the effect of DNA methylation, the increase of DLEC1 expression after TSA treatment (Fig. 7.1C) was more significant than that treated by 5-Aza-dC (Fig. 7.2B). These results suggest that, both DNA hypermethylation and histone hypoacetylation were involved in inhibition of DLEC1 expression; but histone hypoacetylation played a more active role over DNA hypermethylation in this process.

With the above indications, the role of HBV in the DLEC1 epigenetic regulations in HCC cell line was analyzed. Another cell line, HepG2.2.15 was employed to this aim.

HepG2.2.15 is an HBV-expressing and HepG2-derivative cell line [146], considered as a good model to study HBV involved modulation of host cells. The HBV genome expression in HepG2.2.15 cells in our study was confirmed by detecting HBV core gene and surface gene expression on either mRNA or protein level as described in Chapter 4.

Both DNA methylation and histone acetylation status in DLEC1 promoter region was examined in HepG2.2.15. It was found that, the HBV-expressing HepG2.2.15 cells had much lower DNA methylation percentage (Fig. 7.3A) and higher level of histone acetylation (Fig. 7.3B) compared with its original cell line HepG2 (Fig. 7.1A & Fig. 7.2A). However, although the treatment of 5-Aza-dC and TSA changed the DNA methylation (Fig. 7.3A) and histone acetylation (Fig. 7.3B) status significantly, the expression of DLEC1 was not detectable (Fig. 7.3C). This result was not consistent with the results in HepG2 and Huh7 (Fig. 7.1C, Fig. 7.2B). Compared with HepG2, the variation might due to HBV integrated within HepG2.2.15 genome. It is known that the HBV genome integrated in HepG2.2.15 was HBV genotype D [146]. To understand comprehensively the effects of HBV genome on DLEC1 expression, we transfected HepG2 with HBV genotypes A-C and compared DLEC1 expression in these cells (Fig. 7.4B). It was shown in Fig. 7.4B that, different genotypes of HBV affected DLEC1 expression differently. Most significantly, DLEC1 in HepG2 expressing HBV genotype A was up-regulated.

Within HBV genome, HBx has been shown to be a multifunctional protein [193]. The involvement of HBx in epigenetic modifications has also received attention recently [20-22]. Therefore, HBx was chosen as a candidate factor which might be involved in

epigenetic regulations of DLEC1 in HCC cell lines.

We constructed plasmids containing HBx coding sequence with various genotypes, genotype A-D, and transfected them into both HepG2 and Huh7 cells, together with the empty vector pXj40. There was variations in DLEC1 expression between cells expressing different HBx genotypes, and most significantly, HBx A up-regulated DLEC1 expression while HBx D down-regulated it (Fig. 7.5B & C). With this indication, DNA methylation and histone acetylation status of DLEC1 in Huh7 transfected by HBx A-D were further examined. We found that compared with cells transfected by empty vector, there was significant reduction of DNA methylation of DLEC1 in cells expressing HBx A-C, especially HBx A (Fig. 7.6A). For histone H4 acetylation, the acetylation level was increased in cells transfected by HBx A-D, most significant by HBx A (Fig. 7.6B). For HBx B & C, although they also reduced DNA methylation percentage significantly, DLEC1 expression was not affected much (Fig. 7.5B). This might due to two reasons: one is that the histone acetylation level was not increased significantly; and the other is, the contribution of the 19 CpG sites detected might vary from each other, with some sites carrying more importance than other ones. For HBx D, it seemed to have little effect on DNA methylation, although the histone acetylation was increased a bit, DLEC1 expression was still low (Fig. 7.5B). Most significantly, the alteration of DNA methylation and histone acetylation of DLEC1 in cells expressing HBx A was able to be correlated with its expression up-regulation, which indicated that DLEC1 expression was affected by HBx A via both of the epigenetic mechanisms. Our results provided further molecular clues for the clinical observation that, sustained biochemical remission and clearance of

HBV DNA were more frequent in infection of HBV A than other genotypes [184].

The mechanism of genotype-specific effects of HBx on epigenetic alterations of DLEC1 has not been studied yet. However, one of the possible mechanisms is the previously identified proline-arginine-serine rich region (PRS) [36]. As shown in Fig. 7.7, there is significant diversity amongst the four HBx genotypes, especially in the PRS region. In addition to its role in interacting with cytoskeletal proteins [19, 174], further investigation should shed new light on the role of this PRS region in HBx-mediated epigenetic alterations.

Figure 7.7 Alignment of amino acid sequences of HBx genotype A-D. Note that most of the HBx sequences are conserved with majority difference between 30-47 aa, the Proline-Arginine-Serine rich (PRS) region with high variations amongst genotypes.

Conclusively, in the present study, we studied the effects of HBx on regulating the tumor suppressor gene DLEC1 in a genotype-dependent manner. HBx genotype A was found to decrease the DNA methylation and increase histone acetylation levels and

therefore up-regulate DLEC1 expression. Our results have provided new insights on the impact of HBV genotypes on the epigenetic modulations in HBV-mediated hepatocarcinogenesis.

Chapter 8 Overall conclusions and future direction

In conclusion, a cell-based proteomic platform was established in this project. New target proteins associated with HBV-modulated epigenetic changes were identified using this platform, and validated by *in vitro* functional assays. Alterations of expression level in some of the genes have been linked to cancer development, suggesting their tumor suppressor characteristics. Molecular analysis was carried out for two tumor suppressor genes GSTP1 and DLEC1. Our results suggested that HBx affected these TSGs' expression through epigenetic pathways.

8.1 Overall conclusions

8.1.1 Proteomic analysis of target proteins regulated by HBV via DNA methylation

8.1.1.1 Cell-based proteomic platform for epigenetic research

We have combined DNA methylation and proteomic techniques, and developed a new protein profiling platform for identifying target proteins, especially those under epigenetic regulation by HBV.

The term “cell-based” is relative to “clinical sample-based”. The conventional proteomics analysis on HBV-associated HCC has mainly been based on comparing protein profile in cancerous and noncancerous samples [194], including tissues [60, 195, 196] and sera from HCC [126-128]. Results from such a comparative analysis have revealed up- or down-regulated proteins in cancer or non-cancer samples, and their possible correlation with HBV infection.

However, the diversity in results has been reported as a result of the great heterogeneity in clinical samples. Furthermore, the staging of cancer samples selected also impacts on data generated and their interpretation. Therefore, a large number of patients are usually needed for clinical proteomic analysis in HBV-induced HCC to enhance the statistical significance of results.

An alternative approach to avoid the heterogeneity in clinical samples is to use the cell-based system. Such a system is generally cost-effective and more importantly offers a more homogenous genetic background which in turn generates more reproducible data. We are not the first to use a cell-based system. In recent years, HBV-expressing stable cell lines and even transgenic mice have become popular partly due to the homogenous setting and thus enhanced data reproducibility [36, 62, 114]. But we have established this system for the first time to incorporate proteomic analysis and epigenetics. The components are shown in Fig. 8.1.

Figure 8.1 Flow diagram of cell-based platform for analyzing cellular protein profile associated with epigenetic modifications. Treatment of epigenetic inhibitors 5-Aza-dC/TSA incorporates epigenetics into proteomic analysis.

The most important component in this system to incorporate epigenetics into proteomic analysis is the use of epigenetic inhibitor, for example, DNA methyltransferase inhibitor 5-Aza-dC or histone deacetylase inhibitor TSA. For the proteomic analysis part,

there are also other methods besides iTRAQ-coupled 2D LC-MS/MS analysis, for example, another gel-free technique MALDI-TOF/MS, and also the in gel- and LC-based quantitative methods like DIGE and ICAT.

8.1.1.2 Proteomic analysis for target proteins regulated by HBV via DNA methylation

With the developed platform, we compared the proteome expression patterns of cell lines producing (HepG2.2.15) or not producing (HepG2) HBV, as well as both of the cells treated by DNMT inhibitor 5-Aza-dC.

Our findings suggested that HBV replication in HepG2.2.15 cells affected the level of a wide range of cellular proteins possibly involving epigenetic regulation via DNA methylation of the promoter of target genes. Our results confirmed that 15 proteins were down-regulated in HepG2.2.15 but could be reactivated by treatment with 5-Aza-dC, and validated by real-time RT-PCR, Western blots, and bisulfite analysis of DNA methylation patterns.

The identification of 15 proteins provided molecular clues on reported clinical observations related to HBV-associated HCC. This platform was also applied to analyze the effect of HBx genotypes A-C. A number of the identified proteins overlapped with the proteins identified in HBV-producing cells. Our results suggested that HBx may have a direct involvement in epigenetic regulations.

8.1.1.3 Significance and limitations of the results

The expression alterations we identified of these proteins, as well as the DNA methylation status in their promoters, could serve as targets for further mechanistic studies on epigenetic regulation of HBV-associated HCC development. As DNA methylation can occur early in cancer development, the hypermethylation of these genes may serve as a candidate biomarker in early scanning and diagnosis of HCC in populations with higher risks.

Unexpectedly, the previously identified TSGs E-cadherin and p16 by other reports were not found in our proteomics data. This may be explained by a number of possible ways. Firstly, different cells lines may have been used in different studies, which may cause variations in protein profiles identified. Secondly, to mimic the natural way of HBV expression, the expression of the replicative HBV genome was driven by its own promoter, instead of other strong promoters such as CMV promoter. The difference in promoter used can lead to variation in HBV expression level, and therefore affect the proteomic profiles. Thirdly, there may be variations in the conditions of 5-Aza-dC treatment, including the concentration of 5-Aza-dC and the time point that cells were collected before subject to proteomic analysis. Another reason could be the sensitivity of the method, including the total amount of protein used, labeling efficiency, the sensitivity of the LC-MS/MS machine, among others.

8.1.2 Case studies of TSGs epigenetically affected by HBV/HBx

Based on the identified target genes in ours and other published results, the molecular mechanisms involved in HBV/HBx-induced epigenetic regulations were further analyzed. We performed molecular mechanism studies of epigenetic regulations of target genes by HBV/HBx: two tumor suppressor genes, GSTP1 and DLEC1. The genotype-specificity of HBx in epigenetic regulation of these TSGs was also evaluated.

8.1.2.1 HBx inhibits GSTP1 expression in HCC cells

GSTP1 expression was found to be completely depleted in HBV integrated HepG2.2.15 cells as a result of the hypermethylation in its promoter region. HBx genotype D was shown to be involved in the repression of GSTP1. This repression was also confirmed by functional studies including ROS assay and apoptosis detection. Our results provided new evidence that the cellular protection within human liver can be compromised by HBV infection, possibly via HBx, and should facilitate the understanding of HBV and their influences on the epigenetic modulations for epigenetic tumorigenesis during HBV-associated HCC development.

8.1.2.2 HBx regulates DLEC1 expression via DNA demethylation and histone acetylation in HCC cells

HBx genotype A was found to decrease the DNA methylation and increase histone acetylation levels of DLEC1 promoter. These dual mechanisms resulted in up-regulation of the DLEC1 expression.

Taken together, our findings provided new insights on the involvement of HBV/HBx in the epigenetic modulations. These in turn may be helpful for a better understanding of the role of epigenetic modulations in HBV-mediated hepatocarcinogenesis.

8.2 Future direction

Using a cell-based system, our results have provided new information on the involvement of HBV/HBx in the development of HCC via the epigenetic processes. In addition, the genotype-specific effect of HBx has also been demonstrated. However, the tumor suppressor genes selected for mechanism studies were largely selected based on the widely reported TSGs. The involvement of other target proteins identified in our proteomic studies should be further analyzed by molecular biology techniques. Based on our findings of genotype-dependent manner of HBx in regulating GSTP1 and DLEC1, the other factors involved should be evaluated, including DNMTs. Therefore, to gain a more comprehensive and further understanding of the molecular mechanisms of

HBV-associated HCC via the cellular epigenetic processes, we propose the following experimental plan in the future:

8.2.1 Mechanism studies on proteins identified in proteomic analysis

We employed an effective proteomic analysis with LC-MS/MS to identify on the epigenetically silenced genes by HBV/HBx. For those proteins whose changes by HBV/HBx have been confirmed by RT-PCR and Western blotting, mechanism studies such as DNA methylation analysis, cell functional assays (apoptosis assay, colony formation assay, ROS assay, trans-well assay and others), should be followed to give further and deeper understanding of HBV involved epigenetic mechanism of HCC onset.

8.2.2 Further explorations on the epigenetic mechanisms of genotype-specific HBx function in HCC development

8.2.2.1 Full pattern of the modulation of DNMTs by HBx genotypes A-D

It has been suggested that the function of HBx-related DNA methylation is related to the regulation of the expression of DNMTs. However, the role of the genotype has not been analyzed comprehensively so far.

Encouragingly, our data on GSTP1 and DLEC1 suggested that the function of HBx on

DNA methylation may well be genotype-specific. For example, HBx genotype D can significantly inhibit GSTP1 expression via DNA hypermethylation, while the effect of genotype A, B and C can are not significant. Therefore it should be worthy of examining if the pattern of HBx with various genotypes can be related with its function of up-/down-regulating gene expression.

Both the DNMT1 expression and DNMT1 activity should be examined and serves further as a confirmation to the down-stream TSGs changes.

8.2.2.2 Involvement of other factors

Based on our findings of HBx in regulating DNA methylation of GSTP1 and DLEC1, the other factors involved in this process should be evaluated, like DNMTs and MBPs (methylated DNA binding proteins). MBP is another important component of DNA methylation machinery and has been reported to contribute to DNA methylation-induced gene repression [197]. One of the MBPs, MBD2 (methyl-CpG binding domain protein 2) is the only member of MBDs that has been reported to be both a transcriptional repressor [109] and a DNA demethylase [198]. It has been identified that MBD2 represses GSTP1 transcription via hypermethylation in HCC cells [199], but its expression was negatively correlated to p16 promoter hypermethylation status in HCC tissues, according to a clinical observation [200]. This could suggest a gene-specific or promoter-specific role of MBD2 in regulation of its target genes' expression via hyper- or de-methylation [201]. Thus,

whether MBD involved in HBx-mediated DNA hypermethylation remains to be evaluated.

8.2.2.3 Further validation of genotype-specific function of HBx

The genotype specific function of HBx in epigenetically regulating GSTP1 and DLEC1, identified in our cell-based platform, should be further evaluated on other proteins identified in our proteomic analysis. For example, the effect of HBx genotype A-D on other tumor suppressors in both expression and promoter methylation can be examined.

In addition, the significance of DLEC1 up-regulation by HBx will also be discussed on the level of DLEC1's cellular functions.

Taken together, it is to hope that our cell-based system for epigenetic analysis can be expanded into an analytical platform for a wide range of applications, including the analysis of virus-host interactions (biomarkers of infections) and pharmaceutical applications (biomarker for optimized drug dosing).

Abbreviations

2DE: 2D electrophoresis

5-Aza-C: 5-azacytidine

5-Aza-dC: 5-aza-2'-deoxycytidine

AFP: alpha-fetoprotein

ALDH1: Aldehyde Dehydrogenase 1

ANXA2: Annexin A2

AP: Alkaline Phosphatase

ATCC: American Tissue Culture Collection

BGS: Bisulfite Genome Sequencing

BSA: Bovine Serum Albumin

ChIP: Chromatin Immunoprecipitation

CID: collision-induced dissociation

CIP: Calf Intestinal Alkaline Phosphatase

CPS1: Carbamoyl-phosphate synthase 1

DIGE: Difference in-gel electrophoresis

DLEC1: Deleted in Lung and Esophageal Cancer 1

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: Dimethyl Sulphoxide

DNMT: DNA methyltransferase

EDTA: Ethylenediaminetetraacetic Acid

FACS: Fluorescence-activated cell sorting

FBS: fetal bovine serum

FLNB: Filamin B

G418: Geneticin

GSTP1: Glutathione S-transferase P1

HBc Ag: HBV core antigen

HBs Ag: HBV surface antigen

HBV: Hepatitis B Virus

HBx: HBV X protein

HCC: Hepatocellular Carcinoma

His H4: Histone H4

HRP: Horseradish Peroxidase

ICAT: Isotopo-coded affinity tags

iTRAQ: Isobaric tags for relative and absolute quantification

LC-MS/MS: High-performance Liquid Chromatography with tandem Mass Spectrometry

LINE-1: Long interspersed nuclear element-1

MALDI-ToF/MS: Matrix assisted laser desorption ionisation time-of-flight mass spectrometry

MEIA: Microparticle Enzyme Immunoassay

MEM: Minimal Essential Medium

MBP: Methylated CpG Binding Protein

MS: Mass Spectrometry

MSP: Methylation-Specific Polymerase Chain Reaction

NUH: National University Hospital

PCR: Polymerase Chain Reaction

PBS: Phosphate buffered saline

PVDF: Polyvinylidene Fluoride

RASSF1A: Ras association domain family member 1

ROS: Ractive Oxygen Species

RT-PCR: Reverse transcription Polymerase Chain Reaction

S100A6: Protein S100-A6

SOCS-1: Suppressor of cytokine signaling-1

TBHP: Tert-butyl hydroperoxide

TF: Transcription Factor

TKT: Transketolase

TSG: Tumor Suppressor Gene

TSA: Trichostatin A

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