AN INVESTIGATION OF GENOME-WIDE PROMOTER REGION CYTOSINE-PHOSPHATE-GUANINE (CpG) ISLAND METHYLATION PROFILES IN PATIENTS WITH CHRONIC HEPATITIS B VIRUS INFECTION

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DECLARATION OF CANDIDATE

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DEDICATION

I dedicate this thesis to my parents,

Martha Mokgadi Kgatle and Jacob Malesela Monyepao.

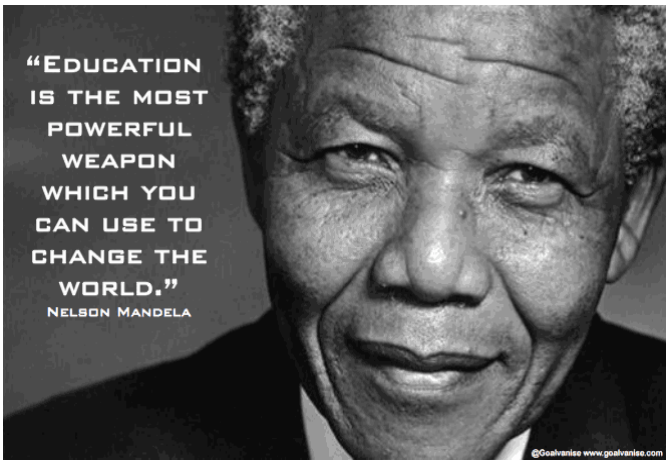
I thank thee Almighty God for giving me life through you.

I am really honoured and proud to call you Mma and Papa.

I love you both dearly.

I also dedicate this thesis to my hero the late

Tata Nelson Rolihlahla Mandela.



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ABBREVIATIONS

α	: Alpha
ß	: Beta
γ	: Gamma
°C	: Degrees Celsius
%	: Percentage
-	: Negative
+	: Positive
µl	: Microlitres
µg	: Microgram
ABHD1	: Abhydrolase domain containing 1
ADP	: Adenosine diphosphate
AGCC	: Affymetrix GeneChip^{R} Command Console
AIB1	: Amplified in breast cancer 1
AIH	: Autoimmune hepatitis
ALAS1	: Aminolevulinate, delta, synthase 1
ALT	: Alanine aminotransferase
ANOVA	: Analysis of variance
APE1	: Human apurinic/apyrimidinic endonuclease I
APC	: Adenomatous polyposis coli
AP	: Activating protein
AP2B1	: Adaptor-related protein complex 2, beta 1 subunit
ARF	: Auxin response factors
ArfGAP	: Auxin response factors -GTPase activating protein
ASPP	: Ankyrin-repeat containing and proline-rich region-containing proteins
ATP	: Adenosine triphosphate
ATP5S1	: ATP synthase H+ transporting mitochondrial Fo complex subunit S
ATRX	: Alpha-thalassemia/mental retardation, x-linked
BARD1	: BRCA1-associated RING domain protein 1
BCAR3	: Breast cancer anti-oestrogen resistance 2
Bcl	: B-cell CLL/ lymphoma
BDS	: Bisulfite DNA sequencing
BIRC3	: Baculoviral IAP repeat-containing 3
bp	: Base pairs
BRCA1	: Breast cancer 1
C6orf97	: Chromosome 6 open reading frame 97
Cp	: BamHI C promoter
cAMP	: Cyclic adenosine monophosphate
CARHSP1	: Calcium regulated heat stable protein 1
CASPR3	: Contactin-associated protein-like 3
CBLC	: Cbl proto-oncogene E3 ubiquitin protein ligase C
CBX8	: Chromobox homolog 8
cccDNA	: Covalently closed circular super-coiled DNA
CCND3	: Cyclin D3
CCDC56	: Coiled-coil domain containing 56 (pseudogene)
CCDC144B	: Coiled-coil domain containing 144B (pseudogene)
CCDC127	: Coiled-coil domain containing 127
CD4^{+}	: Cluster of differentiation 4^{+} T-helper

CDK : Cyclin-dependent kinase
CDKN1C : Cyclin dependent kinase inhibitor 1C
CDKN2A : Cyclin dependent kinase inhibitor 2A
CDH13 : E-cadherin 13
CD21/CR21 : Complement receptor type 21
CFS : Chromosomal fragile sites
CGB8 : Chorionic gonadotropin, beta polypeptide 8
CHML : Choroideremia-like gene
COBRA : Combined bisulfite restriction analysis
COX-2 : Cyclooxygenase 2
CpA : Cytosine-phosphate-Adenine
CpG : Cytosine-phosphate-Guanine
CpT : Cytosine-phosphate-Thymine
CREB : cAMP response element binding protein
CTL : Cytotoxic T lymphocytes
CXorf57 : Chromosome X open reading frame 57
CYB5D1 : Cytochrome b5 domain containing 1
DDB1 : DNA binding protein 1
DEFB109P1 : Defensin beta 109 pseudogene 1
DEFT1P : Defensin theta 1 pseudogene
DGCR11 : Di-George syndrome critical region gene 11
DHBV : Duck hepatitis B virus
DILI : Drug induced liver injury
DLEC1 : Deleted in lung and oesophageal cancer 1
DLR : DNA labelling reagent
DNA : Deoxyribonucleic acid
DNAJC14 : DNAJ (Hsp40) homolog, subfamily C, member 14
DNMTs : DNA methyltransferases
DNaseI : Deoxyribonuclease I
DNASE2B : Deoxyribonuclease II beta
DPC4 : Deleted in pancreatic cancer 4
DPEP3 : Dipeptidase 3
DR1 : Direct repeat 1
DRF : DNA replication foci
DILI : Drug induced liver injury
dsDNA : Double-stranded DNA
DYRK4 : Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 4
E2F : E2 promoter binding factor
E6-AP : E6 associated protein
EBV : Epstein-Barr virus
EBERs : EBV-encoded small RNAs
EBNAs : EBV nuclear antigens
EBP1 : V-erb-b2 erythroblastic leukemia viral oncogene homolog 3-binding protein 1
ECM : Extracellular matrix
EGFs : Epidermal growth factors
EGFR : Epidermal growth factor receptor
EMILIN2 : Elastin microfibril interfacer 2
EMX2 : Empty spiracle homeobox 2
EPHB6 : EPH receptor B6
ErbB3 : V-erb-b2 erythroblastic leukemia viral oncogene homolog 3
ERRC2 : Excision repair cross-complementing rodent-repair deficiency group 2

EXOSC3 : Exosome component 3
FAM106C : Family with sequence similarity 106, member C
FAM173A : Family with sequence similarity 173, member A
FANCE : Fanconi anaemia complementation group E
FXR : Farnesoid x receptor
FasL : Fas ligand
FBXO39 : F-box protein 39
FFPE : Formalin-fixed paraffin-embedded
FGF : Fibroblast growth factor
FGF4 : Fibroblast growth factor 4
FGFRL1 : Fibroblast growth factor receptor-like 1
FRA : Fragile
g : Gram
G : Guanine
GABPB2 : GA binding protein transcription factor beta subunit 2
GCOS : GeneChip^{R} Operating Software
GO : Gene ontology
GK : Lysine and glycine
GRASP : General receptor for phosphoinositides
GSN : Gelsolin
GSHV : Ground squirrel hepatitis virus
GSTP1 : Glutathione S-transferase pi 1
GTPBP5 : GTP binding protein 5 (putative)
HATs : Histone acetylases
HBc : Hepatitis B core
HBcAg : Hepatitis B c antigen
HBeAg : Hepatitis B e antigen
HBs : Hepatitis B surface
HBsAg : Hepatitis B surface antigen
HBx : Hepatitis B x
HBxAg : Hepatitis B x antigen
HBV : Hepatitis B virus
HC : Hepatic cirrhosis
HCC : Hepatocellular carcinoma
HCV : Hepatitis C virus
HDACs : Histone deacetylases
HIST1H4F : Histone cluster 1 H4F
HIV-1 : Human immunodeficiency virus type 1
HLA : Human leukocyte antigen
hMLH1 : Human mutL homolog 1
HPGD : Hydroxyprostaglandin dehydrogenase 15 – (NAD)
HPV : Human xviiiazalxviiiomavirus
HSPA1A : Heat shock 70kDa protein 1A
hTERT : human telomerase reverse transcriptase
HTN3 : Histatin 3
HTR3B : 5-Hydroxytryptamine (serotonin) receptor 3B, ionotropic
Htt : Huntingtin
HSC : Hepatic stellate cells
HYAL3 : Hyaluronoglucosaminidase 3
ICF : Immunodeficiency centromere instability and facial anomalies syndrome
ICR2 : Imprinting center 2

IFIT1B : Interferon induced protein with tetratricopeptide repeats 1B
IFNγ : Interferon gamma
IGFII : Insulin-like growth factor II
IGJBP-3 : Tumour promoter insulin-like growth factor binding 3
IgG : Immunoglobulin G
IITD : Indian Institute of Technology, New Delhi
IKBKB : Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
IKZF3 : IKAROS family zinc finger 3
IL : Interleukin
IL11RA : Interleukin 11 receptor, alpha
IL15RA : Interleukin-15 receptor alpha
IMP-2 : Insulin-like growth factor II mRNA binding protein 2
INR : International normalised ratio
IQR : Inter –quantile range
IRAK2 : Interleukin-1 receptor associated kinase 2
GRID2IP : Glutamate receptor ionotropic delta 2 interacting protein
JAK1 : Janus kinase 1
JNK : c-Jun N-terminal kinase
Kb : Kilobase
KCNK2 : Potassium inwardly- rectifying channel subfamily k, member 2
KCNJ15 : Potassium inwardly- rectifying channel subfamily J, member 15
kDa : Kilo-dalton
KLF1 : Krueppel-like factor 1
KRBA2 : KRAB-A domain containing 2
KRT77 : Keratin 77
KRT42P : Keratin 42 pseudogene
L : Large
LCR : Long control region
LENG1 : Leukocyte receptor cluster member 1
LINE-1 : Long interspersed nuclear element-1
LMP : Latent membrane protein
LNX2 : Ligand of numb-protein X 2
LOC285441 : Uncharacterised
LOC650368 : Asparagine-linked glycosylation 1-like pseudogene
LOC151534 : Ladybird homeobox 2
LOC100189589 : DICTN1antisense RNA 1
LOC401010 : Nucleolar complex associated 2 homolog
LOC729080 : Glycine cleavage system protein H (aminimethyl carrier) pseudogene
LOH : Loss of heterozygocity
LRRC28 : Leucine rich repeat containing 28
LSD : Lysine-specific demethylase
LYPD5 : LY6/PLAUR domain containing 3
M : Middle
MALDI : Matrix assisted laser desorption/ionization
MAT : Model-based analysis of tiling-arrays
MAPK : Mitogen-activated protein kinase
MBP : Methyl-CpG binding protein
MDGA2 : MAM domain containing glycosylphosphatidylinositol anchor 2
MeCPs : Methyl-CpG binding proteins
MeDIP : DNA immunoprecipitation
MEPE : 6kDa matrix extracellular phosphoglycoprotein

mg : Milligram
MGMT : O-6-methylguanine-DNA methyltransferase
Mill2 : Major histocompatibility complex I like leukocyte 2
pMol : Pico molar
ml : Millilitre
mRNA : Messenger RNA
miRNAs : Micro RNAs
MIR644 : MicroRNA 644
MIR138-2 : MicroRNA 138-2
MIR1293 : MicroRNA 1293
MIR573 : MicroRNA-573
MIR527 : MicroRNA-527
MIR4267 : MicroRNA-4267
MS4A13 : Membrane-spanning 4-domains, subfamily A, member 13
MS-SNuPE : Methylation-sensitive single nucleotide primer extension
MSP : Methylation-specific PCR
MTA1 : Metastasis associated 1
mTOR : Mammalian target of rapamycin
MTUS1 : Microtubule associated tumour suppressor 1
ng : Nanogram
N : Number
NAD : Nicotinamide adenine dinucleotides
NADH : Nicotinamide adenine dinucleotides hydrate
NAIF1 : Nuclear apoptosis inducing factor 1
NACC : Nuclear accumbens
NACC2 : Nucleus accumbens associated 2
NAFLD : Non-alcoholic fatty liver disease
NaOH : Sodium hydroxide
NAT6 : N-acetyltransferase 6
NBPF1 : Neuroblastoma breakpoint family member 1
NCBI : National Center for Biotechnology Information
NCF1 : Neutrophil cytosolic factor 1
NCRNA00085 : Non-protein coding RNA 85
NF-κB : Nuclear factor-kappaB
nm : Nanometre
NLS : Nuclear localization signal
NSMAF : Neutral sphingomyelinase activation associated factor
NTCP : Sodium taurocholate cotransporting polypeptide
NTSR1 : Neurotensin receptor type 1
NUDT13 : Nucleoside diphosphate linked moiety X type motif 13
OR52I1 : Olfactory receptor, family 52, subfamily I, member 1
OR5AU1 : Olfactory receptor, family 5, subfamily AU, member 1
ORFs : Open reading frames
OR6C6 : Olfactory receptor family 6, subfamily C, member 6
OR8B4 : Olfactory receptor, family 8, subfamily B, member 4
OR8B8 : Olfactory receptor family 8 subfamily member 8
OR11H2 : Olfactory receptor, family 11, subfamily H, member 2
PA2G4 : Proliferation associated 2G4
PAOX : Polyamine oxidase (exo-N4-amino)
PARD6A : Par-6 partitioning defective 6 homolog alpha
PATE3 : Prostate and testis expressed 3

Abbreviations

PAX1 : Paired box1
pCBPAF : P300/ CREB-binding protein-associated factor
PCDHA1 : Protocadherin alpha -1
PCR : Polymerase chain reaction
PDGFRβ : Platelet derived growth factor receptor beta
P4HTM : Prolyl 4-hydroxylase, transmembrane (endoplasmic reticulum)
PGRF1 : Polycomb group ring finger 1
PGSS : Partek genomic suite software®
PHD : Polybromo-1 homology domain
PI3K : Phophatidylinositol 3–kinase
PLAC8 : Placenta specific 8
PODNL1 : Podocan-like 1
POLA2 : Polymerase (DNA directed) alpha 2
Poly (A) : Polyadenylation
PPAR : Peroxisome proliferator-activated receptor
PPARγ : Peroxisome proliferator-activated receptor gamma
PPP1R8 : Protein phosphatise 1 regulatory subunit 8
PRAME : Preferentially expressed antigen in melanoma
PRAMEF20 : PRAME family member 20
PRMT1 : Protein arginine N-methyltransferase 1
PRSS53 : Protease serine 53
PRSS41 : Protease serine 41
POU5F1P3 : POU class 5 homeobox 1 pseudogene 3
PTEN : Phosphatise and tensin homologue
PTGR2 : Prostaglandin reductase 2
PTOV1 : Prostate tumour overexpressed gene 1
PWWP : Proline-tryptophan-tryptophan proline
RAR-β_2 : Retinoic acid receptor-beta2
RASSF1α : Ras association domain family 1 alpha
RASSF1 : Ras association domain family 1
REC8 : Rec8 homolog
RECQL : RecQ protein-like DNA helicase Q1-like
RGS14 : Regulator of G protein signalling 14
RING-1 : Really interesting new gene domain 1
RMA : Robust multichip averaging algorithm
RNA : Ribonucleic acid
RNA pols : RNA polymerases
ROS : Reactivation oxygen species
RP1 : Retinitis pigmentosa 1 (autosomal dominant)
RRN3P3 : RNA polymerase transcription factor homolog pseudogene 3
RXR : Retinoid x receptor
rtN236T : Asparagines-to-threonine
rTdT : Terminal deoxynucleotidyl transferase, recombinant
SA : South Africa
SAH : S-adenosylhomocysteine
SAM : S-adenosylmethionine
SAPE : Streptavidin-phycoerythrin
Sat-2 : Satellite 2
SCARF2 : Scavenger receptor class F member 2
SCARB2 : Scavenger receptor class B, member 2
SDHA : Succinate dehydrogenase complex, subunit A, flavoprotein

SLC34A1 : Solute carrier family 34 (sodium phosphate) member 1
SNORA7B : Small nucleolar RNA, H/ACA box 7B
SNORA52 : Small nucleolar RNA, H/ACA box 52
SNX11 : Sorting nexin 11
SOC : Stimulate store operated calcium
SPIK : Serine protease inhibitor Kazal
ssDNA : Single-stranded DNA
SSTR1 : Somatostatin receptor 1
STAT : Signal transducers and activators of transcription
SUMO-1 : Small ubiquitin-related modifier-1
TAD : Transcriptional activation domain
Tat : Transactivator of transcription
TBC1D17 : TBC1 domain family, member 17
TBP : TATA binding protein
TBS : Tris-buffered saline
TCEA : Transcription elongation factor A
TF : Transcription factors
TFIIH : Transcription factor II H
TGF-β1 : Transforming growth factor beta 1
THRAP3 : Thyroid hormone receptor associated protein 3
TIRAP : Toll-interleukin 1 receptor domain containing adaptor protein
TLX3 : T-cell leukaemia homeobox 3
TMS1 : Target of methylation-induced silencing
TMEM25 : Transmembrane protein 25
TMEM80 : Transmembrane protein 80
TMEM95 : Transmembrane protein 95
TM6SF1 : Transmembrane 6 superfamily member 1
TNFα : Tumour necrosis factor alpha
TRIM58 : Tripartite motif-containing 58
TRIM77 : Tripartite motif-containing 77
TRIP12 : Thyroid hormone receptor interactor 12
TRPV5 : Transient receptor potential cation channel subfamily v member 5
TSS : Transcription start site
TUBA4A : Tubulin alpha 4a
TUBGCP5 : Tubulin, gamma complex associated protein 5
UBB : Ubiquitin B
UBC : Ubiquitin carrier protein
UBXN1 : UBX domain protein 1
UCT : University of Cape Town
UDG : Uracil-DNA glycosylase
URR : Upstream regulatory region
USA : United State of America
UTR : Untranslated region
XCI : X-chromosome inactivation
Xic : X-inactivation center
Xist : X-inactive-specific transcript
XKR8 : XK, Kell blood group complex subunit-related family member 8
XPB : Xanthoma pigmentosa B
VDRE : Vitamin-D responsive elements
VHL : von Hippel Landau
JAK1 : Janus kinase 1

YDMM : Tyrosine-methionine-aspartate-aspartate

WBV : Woodchuck B virus

WNK2 : Wnk lysine deficient protein kinase 2

Wnt : Wingless type

Wp : BamHI W promoter

ZMYND12 : Zinc finger, MYND-type containing 12

ABSTRACT

Hepatitis B virus (HBV) is oncogenic and a major cause of hepatocellular carcinoma (HCC) in the developing world. It integrates parts of its genome such as the HBx gene, core and surface antigens into the human genome. The integrated viral DNA disrupts gene function resulting in physiological changes that cause liver disease. The viral inserts are inactivated through methylation. This is a protective innate response driven by human DNA methyltransferases triggered by the presence of viral DNA inserts.

This thesis investigates the hypothesis that during the innate response to methylate integrated HBV DNA, there is unintended methylation of genomic DNA around the intercalated viral DNA that could be adjacent host promoter Cytosine-phosphate-Guanine (CpG) islands. This would activate or silence genes including tumour suppressors and result in the clinical disease phenotypes of hepatic inflammation, fibrosis and HCC that characterise chronic HBV infection.

Genome-wide microarray analysis was used to investigate for the presence of promoter CpG island methylation in a cohort of patients with liver disease due to HBV infection, HCC, autoimmune hepatitis which is a non-viral liver disease and normal cases with no liver disease. The study identified hypermethylation in promoter regions, transcription start sites, gene exons and introns. Only sites in the promoter region and within 100bp upstream of a transcription start site were analysed for this thesis presentation. Using an extended cohort of patients with chronic HBV infection and normal controls, bisulfite DNA sequencing was used to validate and confirm the presence of DNA methylation in a selection of some of genes identified.

HBV infected patients were shown to have hypermethylation in the promoter CpG island regions of several genes that regulate hepatic metabolism, tumour suppression, ribonucleic acid splicing, vitamin D receptor binding, protein ubiquitination and the cell cycle. Many of these genes have transcriptional binding factors that are known to be affected by the transcriptional transactivator HBx protein, suggesting that HBx protein is important in the pathogenesis of liver disease.

Amongst the most hypermethylated core promoter regions identified were those for cyclin kinases genes such as *Cyclin D3* (*CCND3*). *CCND3* gene is important in liver regeneration and wound healing and its abnormal function has been linked to the development of liver fibrosis and HCC. Increased methylation of *CCND3* gene was associated with HBV e antigen positive status and genotype D, supporting the hypothesis that increased methylation is associated with host and viral factors.

Methylation induced alteration in the function of the identified gene promoters would affect cellular signalling with effects on cell growth, differentiation, proliferation and apoptosis. These changes would explain the development of hepatic inflammation, apoptosis, fibrosis and malignant transformation seen in chronic HBV infection. Further investigation of these genes will provide new insights on mechanisms of HBV induced liver disease and the development of new molecular diagnostic tools or therapeutic interventions.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. HISTORY OF EPIGENETICS

In the 16th century, the popular theory of preformation implied that a human body developed through the enlargement of a homunculus, which is a diminutive fully formed human being that was thought to exist in the germ cells and merely enlarged into an adult form [1 - 4]. Later in the 17th century, the term "epigenesis" was coined and discredited the theory of preformation. Epigenesis opposed the idea of preformation by suggesting that an embryo develops from successive differentiation of an originally unstructured egg that has been fertilized. In the theory of epigenesis, the formation of all tissues and organs that form a human body proceeded through successive gradual change [3, 5].

The process of epigenesis was thought to be driven by environmental dependent gene expression that differentiated various cell types into functional organs. Conrad Hal Waddington integrated the theory of epigenesis with genetics to propose the term epigenetics. Epigenetics literally means "beyond genetics". The term was used to describe the manner in which the genes interact with their environment to produce a phenotype [6 - 7].

Waddington was a leading geneticist and embryologist of his time who proposed various conceptual models which laid the foundation of the new field of epigenetics. In 1942, he published the book *Principles of Embryology*, in which he presented the term "epigenetics" for the first time in the form of the conceptual model of "epigenetic landscape". He used the metaphor of an epigenetic landscape to describe a surface on which a cell in a developing embryo was represented by a marble. He hypothesised that the development of a cell was influenced by various environmental factors and mutations much like a marble rolling down a series of hills and valleys on the surface or landscape. Consequently, these various environmental factors and mutations would affect the manner in which cells differentiate and develop [6].

The definition of the term epigenetics has been developed further. Currently,

epigenetics is defined as the study of mitotic inheritance and post-translational modification of gene expression, which is not due to any changes in the deoxyribonucleic acid (DNA) coding sequence [8 - 11]. This emphasises alterations that occur during transcription rather than what Waddington described as the "epigenetic landscape".

2. MODERN CONCEPTS OF EPIGENETICS

Epigenetic changes occur largely in multi-cellular eukaryotic organisms and less so in prokaryotes. Prokaryotes are relatively simple and have one single looped chromosome. In contrast, eukaryotes are complicated and have a well-defined nucleus with large quantities of genes and DNA spread across multiple chromosomes [12].

In eukaryotes, DNA is packaged into chromatin within the chromosomes [13, 14]. This protects the DNA from deoxyribonuclease I (DNase1) attack and also ensures that the correct gene is transcribed during transcription. Chromatin consists of DNA structural histone and non-histone proteins [14 - 16]. Within the chromatin, the repeating unit is the nucleosome. Nucleosomes are made-up of 146 base pairs (bp) of two super-helical turns of DNA wrapped around a core containing two copies, each of the histone molecules H2A, H2B, H3 and H4 [16, 13, 17]. Histone molecules are key targets in epigenetics [17]. They have extensive lysine-rich amino terminal tails that protrude from nucleosomes, which make them prone to post-translational modifications [18 - 20].

Eukaryotic histone protein modifications may include acetylation, methylation, phosphorylation, ubiquitination, SUMOylation by small ubiquitin-related modifier-1 (SUMO-1) and adenosine diphosphate (ADP)-ribosylation [15, 21, 22]. These modifications maintain the shape and structure of chromatin, and also affect the availability of transcription factors that are important for gene transcription [15, 18, 23 - 25]. They are catalysed by the activation of several enzymes including DNA methyltransferases (DNMTs), histone acetylases (HATs) and histone deacetylases (HDACs) [21, 24, 26]. Abnormal post-translational histone modifications can cause human diseases through mutations and some of these diseases are summarised in **Table 1.1**.

Table 1.1: Molecular Changes and Biological Effects Caused by Post-Translational Histone Modifications [27 - 40]

Histone Modification	Molecular Change	Some Known Biological Effect
Acetylation	Introduction of acetyl group into an organic molecule	Lysine acetylation leads to toxicity of Htt protein and accelerated Huntington's disease
ADP-ribosylation	Transfer of the ADP-ribose moiety from NAD^+ onto chromatin protein e.g. arginine, aspartic acid	Loss of poly ADP-ribosylation causes progressive neurodegeneration in Drosophila Melanogaster
Methylation	Addition of methyl group to the nucleotide sequence	Loss of X-linked MeCP2 results in Rett syndrome Mutations in the DNMT3B cause autoimmune disease ICF syndrome via hypomethylation.
Phosphorylation	Addition of phosphate group to a protein	Abnormal protein phosphorylation may cause diabetes, arthritis and Alzheimer's disease
SUMOylation	Tagging of SUMO-1 to substrate proteins	Mutations in SUMO-1 site cause familial dilated cardiomyopathy by reducing lamin A sumoylation
Ubiquitination	Transfer of ubiquitin molecule via trans (thiol) esterification reaction	Disruption of ubiquitin E3 ligase in von-Hippel Lindau (VHL) tumour suppressor gene causes VHL syndrome

Abbreviations: **ADP** - Adenosine diphosphate, **DNMT** - DNA methyltransferase, **Htt** - Huntingtin, **ICF** - Immunodeficiency centromere instability and facial anomalies, **NAD** - Nicotinamide adenine dinucleotides, **MeCP2** - Methyl-CpG binding protein 2, **SUMO-1** - Small ubiquitin-related modifier-1, **VHL** - Von Hippel Lindau.

2.1 Epigenetic Regulation of Gene Transcription

DNA methylation and histone protein modifications are important epigenetic events that occur in eukaryotes [41 - 43]. These epigenetic processes affect gene regulation by changing DNA conformation in eukaryotic organisms [12, 43, 44]. Epigenetic changes may influence the expression of several genes during embryonic development [9]. Gene transcription is influenced by *cis*-regulatory elements located within the core promoter, transcriptional factors, methylated DNA sequences and lysine residues within histone proteins [24, 43].

Epigenetic changes determine whether a gene is transcribed or repressed. They are also essential in instructing different cell types in the living organism how and when to read the DNA blueprint. For instance, in the antibody secreting plasma B cells of the immune system, antibody genes are transcriptionally active, but are silenced elsewhere in the body [43].

There are two major ways through which gene transcription can be regulated through epigenetic changes. One way of regulating gene transcription is directly through DNA methylation (see **Figure 1.1**). This involves the addition of a methyl group into DNA sequence. Methyl groups are carbon and hydrogen molecules which bind to the genome through the action of methyl cytosine-phosphate-guanine (CpG) binding

proteins (MeCPs), DNMTs and HDACs, which inactivate gene transcription [45]. Other transcription repressors including nuclear factor kappa B (NF-κB), c-myc/c-myn, activator protein (AP)-2, E2 promoter binding factor (E2F) and the cyclic adenosine monophosphate (cAMP)-response element binding protein (CREB) may also be activated by methyl groups to inhibit gene transcription [20, 46, 47].

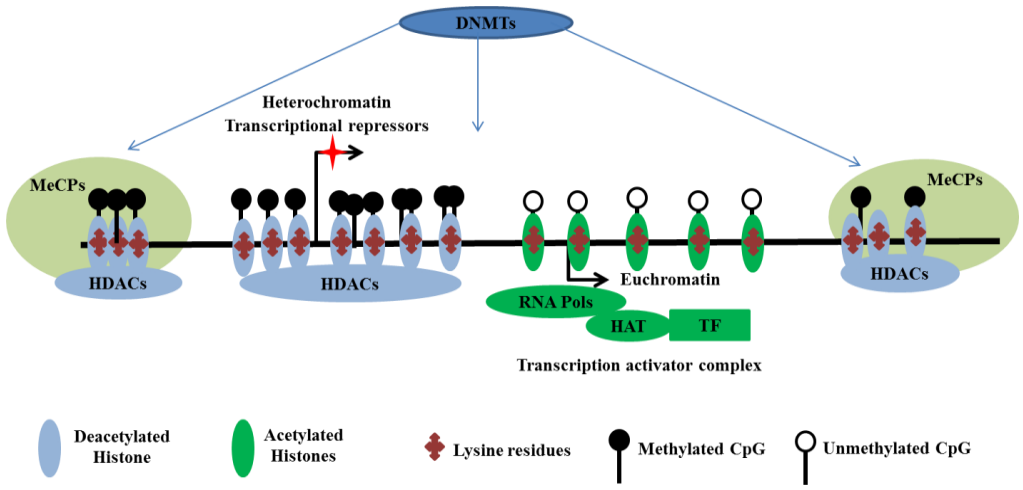

Figure 1.1: The Effects of Epigenetic Changes on Gene Transcription [41- 43].

Chromatin exists in two forms: open - relaxed and closed - folded forms. In an open - relaxed form, the DNA is loose around the histone proteins (open lollipops with green oval circles) and this allows transcription factors to bind the DNA and activate gene transcription (black arrow without a star). Deacetylation of histone molecules (blue oval circles) causes closed-folded form of chromatin (closed lollipops with green oval circles) associated with transcriptional repression (black arrow with a red star).

Abbreviations: **DNA** - Deoxyribonucleic acid, **DNMTs** - DNA methyltransferases, **HAT** - Histone acetylase, **HDACs** - Histone deacetylases, **MeCPs** – Methyl CpG binding proteins, **RNA** - Ribonucleic acid, **RNA Pols** - RNA polymerases, **TF** - Transcription factors.

In addition to DNA methylation, epigenetics can also be regulated by histone protein modifications [18, 45]. Histone protein modifications may be caused by over-expression or aberrant recruitment of HDACs that remodel the chromatin shape and structure (see **Figure 1.1**). The two basic mechanisms responsible for chromatin remodelling are histone acetylation and deacetylation [14, 18, 25, 48, 49]. These mechanisms are controlled by the enzyme activity of HATs and HDACs, respectively [18, 25].

Acetylation of histone proteins is generally acknowledged as playing a key role in gene regulation [18]. For a gene to be transcribed it must become physically accessible to the transcriptional machinery [18, 26 , 25]. Acetylation by HATs substitute the positive charges on the amino terminal tails of histone proteins with an acetyl group derived from acetyl coenzyme A, causing uncoiling of the DNA and euchromatin into an open-relaxed form of chromatin [18, 25]. Consequently, this makes genes accessible to several binding factors such as RNA polymerase II and transcriptional factors, allowing gene expression to occur and proteins to be made (see **Figure 1.1**) [18, 20, 26, 50].

Deacetylation of histone proteins by HDACs results in the tight coiling of the DNA and closed-form of chromatin regions known as heterochromatin [25, 26, 45]. This prevents the interaction between DNA and transcription factors leading to suppression of gene transcription [18]. In some cancer cells, there is increased expression or aberrant recruitment of HDACs and decreased expression of HATs. This results in the hypoacetylation of histone proteins and therefore a condensed or closed chromatin structure [18, 26].

2.2 Role of Epigenetic Changes in Normal Cells

Epigenetic changes are thought to play a critical role during early embryonic development [24, 43, 51]. They maintain the transcriptional repression of inserted viral sequences, non-coding regions, repeat and transposable elements which exist within the genome [52, 53]. Epigenetic changes are also known to either repress or activate certain genes via genomic imprinting and X-chromosome inactivation, although, these mechanisms are regulated differently [54 - 56].

2.2.1 Genomic Imprinting

The seminal work of Surani et al[57] hypothesised that both paternal and maternal genomes are required for normal embryonic development. To test this theory, nuclear transplantation experiments were performed in mice. In these experiments, either the paternal or maternal pronucleus was removed from the fertilized egg and replaced by the opposite sex pronucleus. The obtained uniparental zygote was transferred back to

the mother and the embryo's development was monitored. The results showed that the embryo failed to develop. As a control for these experiments, either maternal or paternal pronuclei were removed and replaced by the same sex pronucleus and this produced a normal embryo. On the basis of their observations, they concluded that both parental contributions are important in producing a healthy normal embryo during development.

Although both parents contribute equally to the normal embryonic development and the genetic content of their off-spring as shown in mice, the genes inherited from each are not equally expressed. This is believed to be due to genomic imprinting. Genomic imprinting is therefore defined as the normal form of epigenetic phenomena that either activates or represses transcription of certain genes based on the parental origin of the DNA. This means that maternally or paternally inherited alleles of a gene are imprinted or marked so that they are monoallelicly expressed [58 - 59].

Surani et al[57] also showed that there were imprinted genes expressed either in maternally or paternally inherited chromosomes which were important for early embryonic development. Three well known classical examples of imprinting in humans are *H19*, *cyclin dependent kinase inhibitor 1C (CDKN1C)* and *insulin-like growth factor II (IGFII)* alleles [60 - 63]. *H19* which is a long non-coding RNA and *CDKN1C* are transcribed only from the maternally inherited allele [61]. The main function of the *H19* maternal allele is to regulate the expression of IGFII protein that is expressed from the paternally inherited allele [62, 63]. The expression of both H19 and IGFII proteins is controlled by imprinting regulatory elements called imprinting center 2 (ICR2) and differentially methylated regions (DMR) [58, 63].

Genomic imprinting is thought to occur through DNA methylation tags and is important in gene regulation by controlling the level of allelic activity required for normal embryonic development in mammals [59]. The process of imprinting is sustained throughout somatic cells as they differentiate into the various cells and organs of an organism. Due to genomic imprinting, the genomes of both parents are distinctively marked with methyl groups that alter the DNA structure as they pass through the germ lines [44, 64]. In turn, germ cells acquire the original biparental epigenetic state based on the sex of transmitting parent [44, 58].

Genomic imprinting occurs in three stages. During the first stage, the imprint from previous generations on both parental genomes is erased in the male and female germ lines, in order to be differentially established once more in the genome of egg and sperm cells [58]. The second stage occurs during gametogenesis and results in the establishment of a new imprint that matches the sex of the transmitting parent. In the final stage, parental imprints are inherited and maintained throughout development [58, 65, 62].

The disruption of genomic imprinting during oocyte growth and development can be detrimental and results in an individual having two active or inactive copies of alleles leading to various human genetic diseases [66 - 69]. Diseases caused by disruption of genomic imprinting include Angelman, Beckwith - Wiedemann, Prada - Willie and Russel - Silver syndromes [66, 67, 69]. Beckwith - Wiedemann and Russel - Silver syndromes arise from mutations of *CDKN1C*, *IGFII* and *H19* genes, which are associated with poor growth of some parts of the body and digestive system abnormalities [69, 70]. Several cancers including cervical carcinoma, hepatoblastoma, human glioma, renal cell carcinoma, colorectal and uterine small-cell cancers have been shown to occur due to abnormal genomic imprinting [69].

2.2.2 Inactivation of X-Chromosome

In 1959, Ohno et al [71] showed that the two female X-chromosomes were different. One chromosome was found to be euchromatic with sparsely packed DNA and active transcription, whereas the other one was heterochromatic, tightly packed and transcriptionally repressed. These observations prompted Mary Lyon in 1962 to hypothesise that one chromosome undergoes inactivation to achieve equal expression levels of X-chromosome linked genes in both males and female [71 - 73].

X-chromosome inactivation (XCI), also known as Lyonisation named after Mary Lyon, results in a monoallelic expression profile whereby one of the two X-chromosomes in females is inactivated. Mary Lyon was intrigued by the fact that female but not male mice had the calico or tortoiseshell coat colour. She performed breeding experiments that demonstrated the inheritance of these colours. An important element of Lyon's hypothesis was the random nature of the inactivation

process, which could therefore affect the maternally inherited or paternally inherited X-chromosomes. This is the reason why male mice could only have one X-chromosome. Lyon's analysis in humans and other species showed that XCI occurs in mammals [54, 55, 73].

XCI occurs at an early stage of development, being initiated at the late blastula stage in mice, and most likely also in humans [73 - 75]. It occurs only in female mammals whereby one of the two copies of X-chromosomes is silenced or activated through DNA methylation. The silenced chromosome turns into a Barr body, which is the process of Lyonization. In addition, the silenced X-chromosome becomes condensely packaged and therefore results in traits like calico or tortoiseshell colour in female cats. A similar process cannot occur in male mammals because they have only one X-chromosome that is required for their survival [73].

The process of XCI occurs through three distinct phases of initiation, spreading and maintenance. Initiation occurs during the early stage of embryonic development [72]. When one of the two chromosomes is randomly activated, the other one remains silenced. Spreading is the process of compacting and inactivating the X-chromosome by a non-coding RNA gene designated *X-inactive specific transcript* (*Xist*) [72, 76, 77]. *Xist* gene binds in the X-inactivation center (Xic) and spreads around the entire X-chromosome in *cis*, resulting in transcriptional repression of one of the two X-chromosomes in the female through epigenetic marking [77]. The repressed chromosome then turns into a Barr body. After the Barr body is formed, maintenance begins and continues throughout adult life. The Barr body remains highly compacted and is essentially inactive through the entire life.

The chromatin state of *Xist* gene is controlled by *Tsix* gene, which acts as an antisense partner of *Xist* [78]. *Tsix* gene is transcribed on the opposite strand and maintains the *Xist*-mediated chromosome silencing by controlling its expression [79 - 81].

2.3 DNA Methylation Changes in the Human Genome

DNA methylation is an important feature of the mammalian genome. It is defined as the attachment of a methyl group (CH_3) to the carbon-5 position of the cytosine ring in the nucleotide sequence (see **Figure 1.2**) [24, 82 - 84].

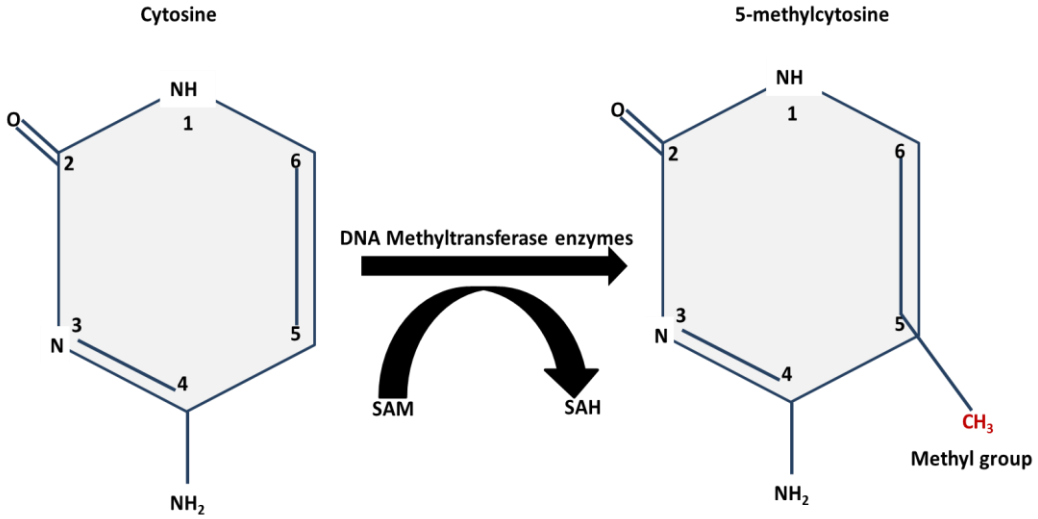

Figure 1.2: DNA Methylation at the Carbon 5 Position of Cytosine (Adapted from Bestor [86]).

The DNA methyltransferase enzyme transfers the methyl group (CH_3) of a SAM to C5 cytosine, yielding 5-methylcytosine and SAH, respectively.

Abbreviations: **C5** - Carbon 5, **CH_3** - Methyl group, **N** - Nitrogen, **NH** - Nitrogen hydrogen, **O** - Oxygen, **SAH** - S adenosylhomocysteine (SAH), **SAM** - S adenosylmethionine.

Eukaryotic DNA methylation changes are established and maintained throughout life by DNA methyltransferases (DNMTs) [85]. DNMT enzymes catalyse the transfer of a methyl group under the influence of a methyl donor S-adenosylmethionine (SAM) leading to the formation of 5-methylcytosine and S-adenosylhomocysteine (SAH, see **Figure 1.2**) [21]. DNA methylation patterns are inherited from the parent strand to the newly synthesized daughter strand during cell divisions [24, 84].

DNA methylation occurs primarily in the context of cytosine-phosphate-guanine (CpG) dinucleotides that are located in CpG islands which are 500 to 5000 bp long. The CpG to guanine -phosphate-cytosine (GpC) dinucleotides are present with the observed-to-expected ratio of 0.65 and G+C content of more than 60%. This is

calculated using the formula (Number of CpG)/ (Number of C x Number of G) x (Total number of nucleotides in the sequence) [24, 84].There are more than 29 000 CpG islands in the human genome [83, 84, 87, 88]. The CpG dinucleotides that are not associated with these islands are highly methylated with 80% being located within the coding region of a gene [83, 84].

In the DNA promoter regions of normal cells, the CpG dinucleotides are protected from DNA methylation regardless of the transcriptional state of genes [89, 90]. However, in cancer cells, the CpG dinucleotides in CpG islands of the gene promoter regions are highly methylated and this correlates with the transcriptional inactivation of genes including genes that are critical for tumorigenesis [89]. In somatic cells, cytosine methylation has also been observed within Cytosine-phosphate-Adenine (CpA) and Cytosine-phosphate-Thymine (CpT) dinucleotides and this methylation also affects embryonic development but to a lesser extent compared to CpG islands methylation [91].

2.4 Physiological Activities of DNA Methyltransferases

DNA methylation profiles are established during early embryonic development by the action of DNMTs [23, 24]. DNMTs have two parts, a diversified amino terminal region and a relatively conserved carboxy terminal region. They have distinctly different methyltransferase activities which are used to categorise them as maintenance DNMTs or *de novo* DNMTs (see **Figure 1.3**) [24, 84].

Maintenance methyltransferase, DNMT1, was identified first in the eukaryotes. It was classified as a maintenance methyltransferase enzyme because it has a 5 to 30 fold preference for hemi-methylated dsDNA over unmethylated dsDNA in *in vitro* [24, 84]. It is responsible for maintaining DNA methylation patterns that *de novo* methyltransferases establish from parental strands to newly synthesised daughter strands throughout cell replication [92]. DNMT1 consists of an amino terminal regulatory and carboxy terminal catalytic domains (see **Figure 1.3**). These domains are connected by a lysine and glycine rich (GK) repeat sequence and mediate protein-protein interaction during chromatin regulation.

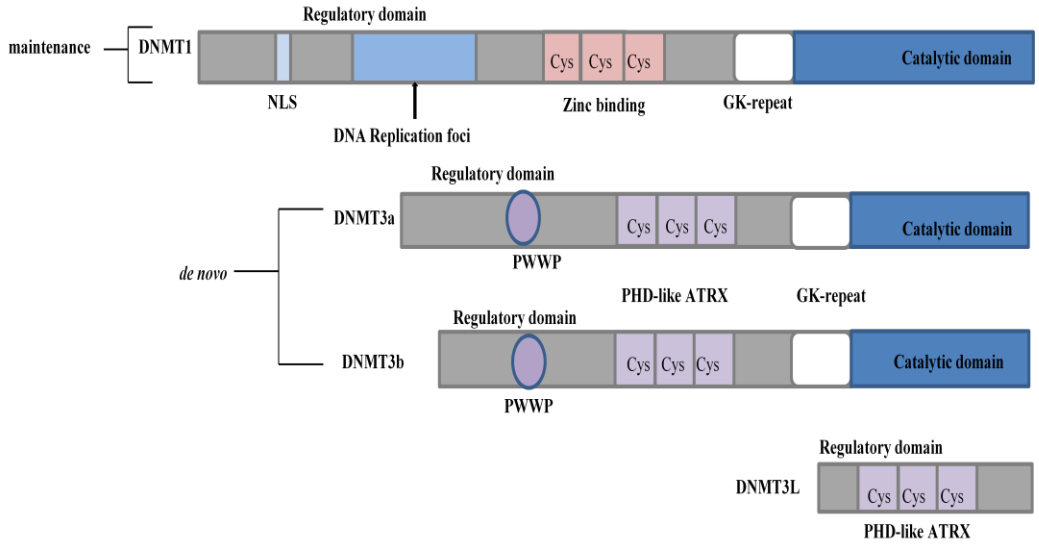

Figure 1.3: The Structure of DNA Methyltransferase Enzymes (Adapted from Robertson and Wolffer[98]**).**

DNMT1 does maintenance methylation and DNMT3A, 3B do *de novo* methylation. They have amino terminal regulatory and carboxyl terminal catalytic domains. The regulatory domain of DNMT1 is longer than that of DNMT3A and 3B. Regulatory domain of DNMT1 has NLS, DNA replication foci, zinc binding cysteine-rich domain and GK-repeat region. In the sequences of DNMT3A, 3B and 3L are PWWP, PHD-like ATRX with a cys-rich region and catalytic domain. In contrast, DNMTL lacks catalytic activity and PWWP, but has PHD-like ATRX region.

Abbreviations: **ATRX** - Alpha-thalassemia/mental retardation, x-linked, **DNMTs** - DNA methyltransferases, **GK** - Glycine lysine, **NLS** - Nnuclear localization signal, **N terminal** - Amino terminal, **PHD** - Polybromo -1 homology domain, **PWWP** - Proline tryptophan tryptophan proline.

The amino terminal regulatory domain has a nuclear localization signal (NLS), DNA replication foci (DRF) and cysteine-rich region which binds to zinc ions [93]. The NLS sequence is responsible for DNMT1 localization in the nucleus where it is recruited to DRF during the S phase of the cell cycle and preferentially methylates hemi-methylated cytosines [93]. This occurs through interaction between DNMT1, ubiquitin-like containing polybromo-1 homology domain (PHD) and really interesting new gene finger domain 1 (RING-1) [93, 94]. RING-1 finger domain 1 is a protein structural domain that binds one or more zinc ions in the DNA sequence. It plays an important role in chromatin remodelling and gene transcription [95, 96]. Disruption of interaction between DNMT1, PHD and RING-1 promotes the development of tumour in human and glial cells [97].

In contrast to maintenance DNMT1, the regulatory domain amino terminal regions of *de novo* methyltransferase enzymes are poorly conserved and smaller, but their catalytic domains are well conserved [21, 49]. In the centre between regulatory and catalytic domains of *de novo* methyltransferase enzymes are proline-tryptophan-tryptophan-proline (PWWP) and PHD-like alpha-thalassemia/mental retardation, x-linked (ATRX) cysteine-rich regions (see **Figure 1.3**). The *ATRX* gene is located on the X-chromosome and regulates adenosine triphosphate (ATP)-dependent chromatin remodelling. Mutations in the gene cause X-linked mental retardation syndrome that occurs mainly in males due to X-linked recessive inheritance [21, 49].

DNMT3A and 3B are *de novo* methylatransferases [85]. They establish the DNA methylation patterns of dsDNA during embryonic development [64]. They were mapped on chromosomes 2p23 and 20q11.2, respectively [99, 100]. The DNMT3L enzyme is located on chromosome 21q22.3. Its methyltransferase enzyme structure is similar to that of DNMT3A and 3B, but its biological catalytic activity remains unknown. DNMT3L increases the ability of DNMT3A and 3B to bind to DNA and stimulates their *de novo* methyltransferase activities. It interacts directly with DNMT3A and 3B to establish maternal *de novo* methylation during genomic imprinting [24, 84, 85].

The biological effects of both maintenance and *de novo* methylations have been characterised [101]. The DNMT1 is highly expressed in sperm, suggesting a role during spermatogenesis [51]. In early embryogenesis, DNMT1 enzyme maintains the methylation patterns of genomic imprinted genes and its absence or over-expression is associated with embryonic lethality. DNMT3B is highly expressed in bone marrow, thyroid and testicular tissues, but less so in brain, skeletal muscle and peripheral blood mononuclear cell [100 - 102]. Mutations in the DNMT3B gene are associated with increased chromosomal rearrangements in methylated centromeric regions that may lead to the development of immunodeficiency centromere instability and facial anomalies syndrome (ICF) [28, 29, 42] . These mutations can also impair the regulation of DNMT3B by DNMT3L [41].

DNMT enzymes can be targeted by tissue-specific micro RNAs (miRNAs) which are small ribonucleic acids of 18 to 25 nucleotides in length [103]. In human cancers, various miRNAs are lined up with the untranslated region (UTR) that binds to

DNMTs and induce translation repression and degradation of messenger RNA (mRNA). MiRNA-148 down-regulates DNMT1 leading to over-expression of methylation-sensitive genes in cluster of differentiation 4^+ T-helper ($CD4^+$) cells and silences the *ras association domain family 1 alpha* (*RASSF1α*) gene in human cholangiocarcinoma xenografts [104, 105]. Interaction of miRNA-29 family members with 3'UTR of *DNMT3A* and *DNMT3B* genes has been shown to result in global DNA hypomethylation in lung cancer cells [103, 104].

2.5 DNA Methylation and Cancer Development

Cancer develops as a result of germline mutations and aberrant DNA methylation [106]. Cancer is associated with the activation and silencing of proto-oncogenes and tumour suppressor genes, respectively [48]. According to Knudson's two-hit hypothesis, the functions of both alleles in the tumour suppressor gene must be altered in order for the gene to lose its function completely and result in cell transformation. Germline mutations can silence tumour suppressor genes, resulting in the loss of normal gene function. Therefore, promoter hypermethylation could be considered as a second hit after germline mutation [107, 108].

Cancer cells usually have significant aberrant DNA methylation changes that affect genes involved in the prevention of carcinogenesis. Tumour suppressor genes and oncogenes play a vital role in controlling cell proliferation, programmed cell death and the repair of damaged DNA [41, 42]. Any disruption of their functions may result in malignant transformation leading to cancer [41, 109].

Cancer associated DNA methylation may be global hypo- or hypermethylation (see **Figure 1.4**). Global DNA hypomethylation was the first epigenetic abnormality identified in cancer by methylation-sensitive restriction enzymes [41, 47]. Hypomethylation involves a decrease in the methylation of CpG dinucleotides in DNA and causes increased mutation rates [92]. It may contribute to carcinogenesis by causing loss of genomic imprinting, X-chromosome inactivation and induction of genomic instability through transcriptional controls.

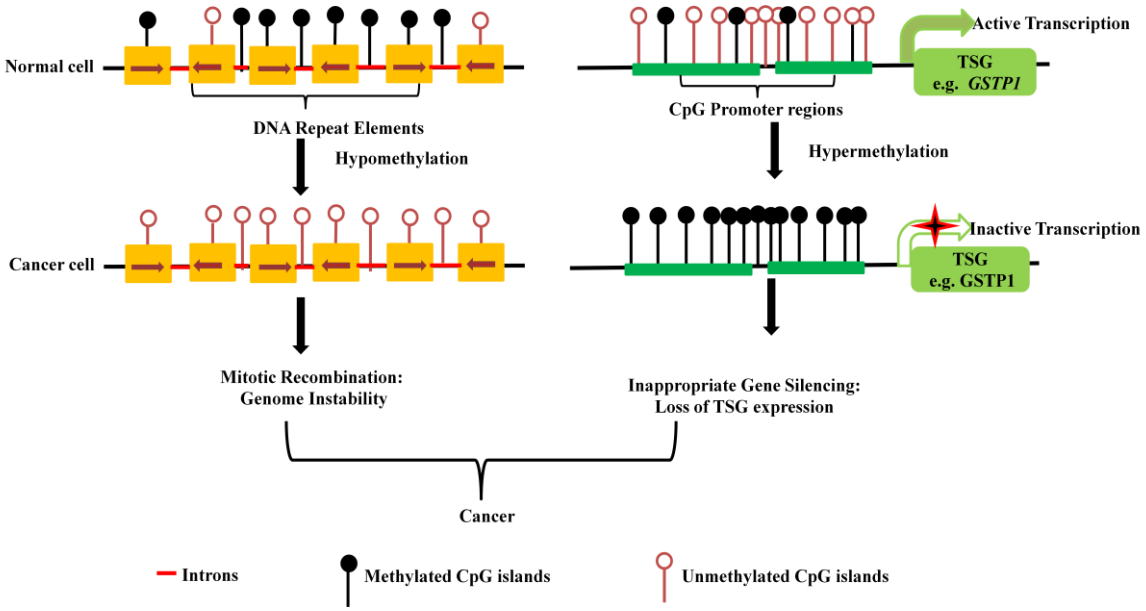

Figure 1.4: DNA Methylation in Normal and Cancer Cells (Adapted from Perret[112]).

Most CpG sites within the genome of the normal cell become stably methylated (as shown by closed lollipop) for normal embryonic development. Simultaneously, some CpG sites within the promoter regions are also unmethylated (as shown by open lollipop) and this is associated with normal active transcription of tumour suppressor genes important to prevent the development of cancer. In cancer cells, the CpG sites outside the CpG islands become hypomethylated, while the CpG dinucleotides within the promoter regions of tumour suppressor genes which are usually not methylated in normal cells are associated with hypermethylation leading to silencing of gene transcription.

Abbreviations: **CpG** - Cytosine-phosphate-Guanine, **GSTP1** - Glutathione S-transferase pi 1 gene, **TSG** - Tumour suppressor gene.

Hypomethylation of DNA may also target the transposons, the repeated DNA elements and introns in which they activate the encoded genes, preferably proto-oncogenes, which are normally transcriptionally repressed in normal cells [41, 42, 109]. This abnormality is observed in a wide variety of cancers and is seen in neoplastic nodules. For example, Long interspersed nuclear element 1 (LINE-1) repeats, *epidermal growth factor receptor* (*EGFR*), *fos*, *raf*, *myc*, *ras*, and *wnt* genes are hypomethylated and down-regulated in hepatocellular carcinoma (HCC), breast, colorectal, colon, gastric and many more cancers [41, 47, 110, 111].

Hypermethylation appears in the early and late stages of carcinogenesis [42, 48]. CpG sites that are usually unmethylated in normal cells become hypermethylated, resulting in the transcriptional repression of the encoding tumour suppressor genes

and the development of cancer [112] (see **Figure 1.4**). It is not known why some CpG sites are hypermethylated while others are not. Genes usually known to be hypermethylated in human cancers generally play important roles in cell-cycle regulation, cell adherence, metastasis, apoptosis, repair and protection of DNA [19, 113, 117]. **Table 1.2** illustrates a list of genes that are usually hyper- or hypomethylated in various tumours.

Table 1.2: Genes Methylated in Human Cancers and Their Role in Tumour Development [32, 47, 48, 110, 111, 113, 114, 117, 119 -126, 128 - 134, 442 -443]

Gene	Tumour Type	Role in Tumour Development
Hypomethylated		
β-catenin	HCC, Parathyroid carcinoma, Colorectal and Breast cancers	Wnt signaling
EGFR	HCC, Breast, Lung, Anal cancers	Cell-cycle regulation
Myc	Breast, Cervical, Colon and Lung cancers	
Fos	Breast, Thyroid and Ovarian cancers	
Ras	Adenocarcinoma, Thyroid, Lung and Colon cancers	
Raf	Gastric, Leukaemia, Thyroid and Prostate cancers	
LINE-1	HCC, Colon, Bladder, Familial and Sporadic cancers	Apoptosis and DNA damage
Hypermethylated		
APC	Prostate, Colorectal, Breast and Esophageal cancers	Cell-cycle regulation
CDKN2A	Lung, Colorectal, Head and Neck cancers	
DLEC1	HCC, Breast, Lung and Esophageal cancers	
pRb	Colon, Oligodendroglioma, Retinoblastoma cancers	
$p16^{INK4\alpha}$	Gastric, Breast, Head and Neck, Colorectal, and Lung cancers	
$p15^{INK4}$	Gastric, Leukaemia, Lymphoma, Liver and Lung cancers	
$p14^{ARF}$	Gastric, Prostate, Bladder	
RASSF1	Prostate, Colorectal, Ovarian and Nasopharyngeal cancers	
RARβ	HCC, Esophageal carcinoma and Prostate cancer	
VHL	Renal cell cancer and Esophageal carcinoma	
BRCA1	Breast and Ovarian cancers	Repair and protection of DNA
GSTP1	Gastric, Prostate HCC and Breast cancers	
hMLH1	Gastric, Colon and Ovarian cancers	
MGMT	Gastric, Head and Neck, Colorectal and Brain cancers	
DAPK	Lung, Brain, Head and Neck cancers	Apoptosis
E-cadherin (CDH13)	HCC, Breast, Prostate, Cervical, Head and Neck cancers	Cell adherence and metastasis
ERα	Breast, Colon and Prostate cancers	Hormone resistance

Abbreviations: **β** – Beta, **APC** -adenomatous polyposis coli, **BRCA1** - Breast cancer 1, **CDKN2A** - Cyclin dependent kinase inhibitor 2A, **CHD13**- coronary heart disease 13, **DAPK** - Death associated protein kinase, **DLEC1**- Deleted in lung and oesophageal cancer 1, **DNA** - Deoxyribonucleic acid, **EGFR** - Epidermal growth factor receptor, **ERα** - Estrogen receptor-alpha, **GSTP1** - Glutathione S-transferase pi 1, **HCC** - Hepatocellular carcinoma, **hMLH1** - Human mutL homolog 1, **LINE-1** - Long interspersed nuclear elements, **MGMT** - O-6-methylguanine DNA methyltransferase, **RASSF1**- Ras association domain family 1, **pRb** - Retinoblastoma protein, **RARα** - Retinoic acid receptor alpha, **VHL** - Von Hippel Landau, **Wnt** - Wingless type.

3. ONCOGENIC VIRUSES AND DNA METHYLATION

Worldwide, 20% of human cancers are linked to infection with oncogenic viruses [135, 136]. Three well-studied viruses associated with malignant transformation are human papillomavirus (HPV), hepatitis B virus (HBV) and Epstein-Barr virus (EBV). These hepadnaviruses are capable of establishing persistent infection which in a small minority of those infected may give rise to cancer [137 - 139].

The EBV, HPV and HBV infections are associated with gastric or nasopharyngeal, cervical and liver cancers respectively [139]. The various mechanisms by which these viruses cause malignancy are still under investigation, but hypermethylation has been shown to be one such mechanism. The cellular methylation systems that are activated during chronic viral infections methylate the viral genome inserted in the human genome as an innate protective mechanism to stop viral replication [135, 140, 141]. However, the same methylation system may also methylate and suppress important tumour suppressor genes and oncogenes, resulting in malignant transformation that may lead to the development of cancer [142].

3.1 Epstein - Barr Virus and DNA Methylation

EBV or human herpesvirus 4 is a DNA virus that was first described in Burkitt's lymphoma B-cells by British pathologists Michael Anthony Epstein and Yvonne Barr [143]. It was the first oncovirus to be identified and is also known to be associated with Hodgkin's lymphomas, gastric and nasopharyngeal carcinomas [116, 142, 144 - 150]. It is classified into EBV-1 and EBV-2, which differ in the sequence of the genes that code for the viral genome [150 , 151].

About 90% of the adult population worldwide is infected with EBV. The infection occurs mostly during childhood without showing any evidence of symptoms. EBV is transmitted from one individual to another through intimate contact with saliva from an EBV infected person. It infects the B-cells which express solely complement receptor type 21 (CD21/CR21) [147, 152, 153]. However, it may also infect nasopharyngeal epithelial cells [144].

The primary infection of EBV *in vitro* begins through interactions of envelope glycoproteins gp350/220 with CD21 receptor and gp21 with human leukocyte

antigen (HLA) class II co-receptor [152 - 154]. These interactions trigger fusion and internalization of the virus membrane with the cell membrane. Following endocytosis of the virus into the host cell, the EBV genome circularises to exist in an episomal form. Episomes replicate simultaneously with the cellular DNA replication and establish latent infection [153, 155]. During latent infection, various EBV genes including EBV nuclear antigens (EBNAs), the latent membrane proteins (LMPs) and the EBV-encoded small RNAs (EBERs) are expressed [156, 158].

EBV is a gammaherpesvirus of the *Lymphocryptovirus* genus. It is composed of a toroid-shaped protein. It has 0.5kb terminal direct repeats at both ends of a linear genome that divide the genome into short and long sequence domains. Its genome contains linear, double-stranded DNA (dsDNA) of 172 kilobases (kb) in length[159]. It also has various open reading frames (ORFs), which encode various EBV genes that are located on the BamHI restriction fragment. The BamHI restriction fragment is made-up of four viral promoters including BamHI W promoter which play an important role in the transcription of viral gene expression [152, 153].

The BamHI W promoter of EBV is susceptible to DNA methylation and this alters the expression of EBNA proteins [115, 147, 152, 153, 160]. Methylation of the EBV genome induces aberrant methylation that suppresses the transcription of important host tumour suppressor genes and leads to uncontrolled cell growth [161]. The activation of oncogene latent membrane protein (LMP) 1, one of the LMP proteins, enhances upregulation of DNMT1, which is associated with hypermethylation of *retinoic acid receptor-beta2* (*RAR-β_2*) and *E-cadherin 13* (*CDH13*) genes in EBV-associated nasopharyngeal cancer [121, 157].

The activation of DNMT1 by LMP2 causes the suppression of LMP2 gene expression that results in the hypermethylation of the phosphatase and tensin homolog (PTEN) promoter in EBV-related gastric cancer. EBV-negative gastric cancer does not have such hypermethylation changes [162].

EBV-induced gastric adenocarcinoma is associated with promoter methylation of *p14, p16* and *adenomatous polyposis coli* (*APC*) genes [115]. Other EBV-driven aberrant CpG islands promoter hypermethylation in human gastric cancer include *EPH receptor B6* (*EPHB6*), *MAM domain containing glycosylphosphatidylinositol anchor 2* (*MDGA2*), *interleukin-15 receptor alpha* (*IL15RA*), *scavenger receptor*

class F member 2 (*SCARF2*), *somatostatin receptor 1* (*SSTR1*) and *Rec8 homolog* (*REC8*) [163].

3.2 Human Papillomavirus and DNA Methylation

Worldwide, cervical cancer is the second most common cancer affecting women. Approximately 500 000 women are diagnosed each year with cervical cancer and almost 300 000 die from the disease [164, 165]. Nearly all cases of cervical cancers are caused by HPV, with about 630 million people infected worldwide [138, 166 - 169].

More than hundred types of HPV have been characterised and are classified into low- or high-risk HPV types [168]. HPV -1, -6 and -11 are low-risk types that cause foot and genital warts. They are found mostly in benign and low grade squamous intraepithelial lesions [138, 168, 169]. High-risk HPV types 16, 18, 31 and 45 cause cervical cancer, and are found in high grade squamous intraepithelial lesions [168, 170 - 174]. The best described types are HPV-16 which is associated with squamous cell carcinoma and HPV-18 associated with adenocarcinoma [168, 175].

The transmission of HPV occurs mainly through direct skin-to-skin contacts such as during anal and vaginal intercourse. The life cycle of HPV begins with infection of epithelial basal cells. Following infection and uncoating of the viral capsids, the viral genome is transcribed into the nucleus. In the nucleus, the HPV genome exists as an episome, which replicates in order to establish persistent infection [169, 176 - 178].

HPV is a non-enveloped dsDNA virus of the genus *Alpha-Papillomavirus* in the *Papillomaviridae* family [173, 175, 176]. HPV genome is approximately 8 kb in length and contains eight ORFs which encode E1-E7 and Late (L) proteins L1 and L2 [178 - 181]. An upstream regulatory region (URR) of HPV genome is 850bp long control region (LCR) of HPV genome. The URR contains a TATA box and Sp1-binding sites and is therefore important for initiating replication and gene transcription regulation [182, 183].

Infection with high-risk HPV types is associated with integration of viral DNA into host DNA. HPV integration in the human genome has been linked to disruption of viral E2 ORF that results in the loss of E2 protein [181]. E2 protein regulates the expression of E6 and E7 oncogenes [180]. The loss of E2 protein may result in the transcriptional repression of E6 and E7 proteins [182, 184].

In some HPV infected cells, ORF E2 over-expresses early viral proteins E6 and E7, which extend the cells' life span [179, 185]. These cells retain the capacity to proliferate a process known as immortalization [186]. During cell immortalisation caused by HPV, normal cellular mechanisms that protect cells from mutations are disabled and selective tumour suppressor genes are inactivated. As mutations accumulate, the cells undergo morphologic or epigenetic changes that lead to the development of precancerous cells and cancers of the cervix, vagina or vulva [161, 172, 186, 187].

Epigenetic changes in HPV occur through the interaction of viral proteins with host epigenetic machinery proteins such as DNMT and HAT [184, 188]. For instance, HPV-16 E7 protein binds to DNMT1 and P300/ CREB-binding protein-associated factor (pCBPAF) acetyltransferase and regulates their enzymatic activities [93, 189]. HPV-16 E6 protein interacts with transcriptional activator pCBPAF or p53 and inhibits HAT activity. Interaction of ORF E6 protein with p53 promotes cell cycle progression and therefore enhances viral replication [161, 184]. HPV type 16 and 18 E6 proteins also bind and inactivate human telomerase reverse transcriptase (hTERT) protein through proteasomal degradation by ubiquitin ligase E6 associated protein (E6-AP) [186, 187, 190].

DNA methylation also occurs in HPV -16 and -18 infections [191 - 194]. In HPV-16 related invasive cervical cancer, LCR and E2 binding sites at the URR are hypermethylated [137, 193]. The methylation of these binding sites disrupts the function of E2 protein resulting in the upregulation of E6 and E7 oncogenes [192, 193, 195, 196]. Similar patterns of methylation were observed in immortalised cells from foreskin keratinocytes and primary cell carcinomas transfected with HPV-18 [188, 191, 194, 197].

3.3 Hepatitis B Virus and DNA Methylation

HBV was first identified in the 1960s and was the first human hepatitis virus to be well characterised at a molecular level [198]. HBV is classified as an *Orthohepadnavirus* which belongs to the family *Hepadnaeviridae*. Contained in this family, are other viruses such as the hepatic viruses of woodchucks, ducks, herons, ground and tree squirrels**.** These viruses were classified as *Hepadnaeviridae* due to their structure and genomic organization being similar to that of HBV. They replicate via reverse transcription of RNA intermediates, the step in which the DNA is

packaged into hepadnaviral infectious particles [199 - 204].

HBV infection may be acute or chronic. Acute infection occurs shortly after the initial exposure and it may take up to 8 weeks for symptoms to show [205 , 206]. Chronic infection is defined as the presence of hepatitis B surface antigen (HBsAg) in the blood more than 6 months following initial infection. It results in the development of liver cirrhosis and hepatocellular carcinoma (HCC) [205, 207 - 209].

Epidemiological studies show that chronic HBV infection is a major cause of liver cirrhosis and HCC [210 - 213]. HCC, also known as malignant hepatoma, primary liver cell carcinoma or hepatocarcinoma, is the third leading cause of global cancer-related deaths after colon and lung cancer [210, 214].

The link between HBV and HCC was identified a decade after the discovery of HBV infection by Sherlock, who noticed a high prevalence of chronic HBV infection in HCC cases [215]. HCC occurs in individuals who have chronic HBV infection. Progression from acute to chronic HBV infection is common in infants and children with an estimated rate of 70 – 95 % due to an immature immune system while only about 3 – 5 % of acutely HBV infected adults may develop chronic infection [210, 216 , 217].

The progression of HBV infection to HCC occurs in a series of steps generally following a sequence of chronic HBV infection, fibrosis or cirrhosis, dysplastic nodule formation and HCC development [218 - 220] as illustrated in **Figure 1.5**. It is thought that during this sequence progression, mutations and epigenetic alterations accumulate and lead to the development of malignant transformation in the HBV infected liver [207, 221, 222]. It has been reported that 10 to 20 % of chronic HBV infected patients may develop cirrhosis within a period of 3 to 5 years [218]. The average period of HBV infection progression to cancer is about 20 to 30 years and 8 to 10 years after the development of cirrhosis [207, 216].

Aberrant DNA methylation of promoter CpG islands is the primary epigenetic change seen during the course of HBV infection as it progresses to cirrhosis and HCC. Such methylation is detected at higher rates in HCC cells and less so in hepatocytes at the stage of chronic HBV infection, fibrosis and cirrhosis. This suggests the important role of epigenetic-related alteration in liver disease.

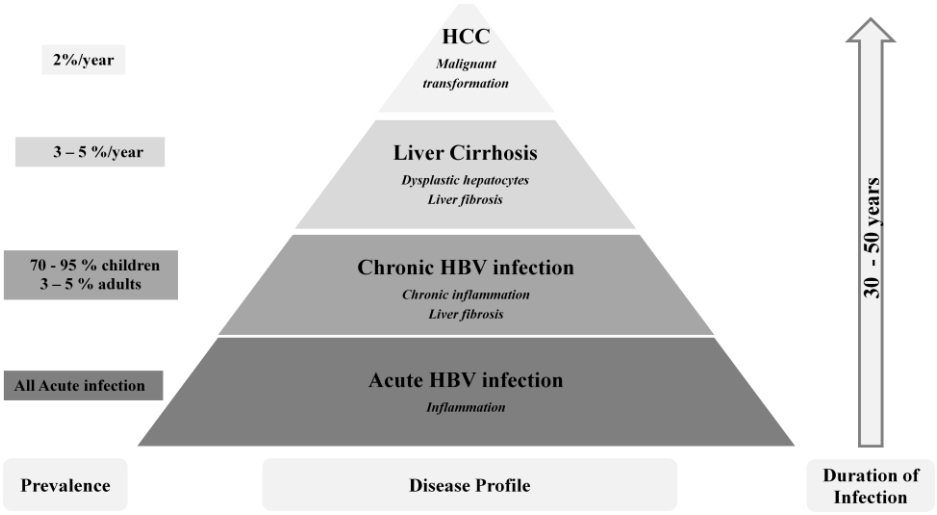

Figure 1.5: Chronic HBV Infection, Cirrhosis and HCC Development.

About 70 - 95 % of children and 3 - 5 % adults infected with HBV may progress to chronic HBV infection associated with chronic inflammation and fibrosis.

Abbreviations: **HBV** - Hepatitis B virus, **HCC** - Hepatocellular carcinoma.

3.3.1 Transmission of HBV

HBV can be stable for seven days or more on dry environmental surfaces. The two major routes of HBV transmission are horizontal and perinatal or vertical transmission. The efficient modes of transmission are blood and sexual contact with an infected person [223 - 225].

The virus is horizontally transmissible during child to child physical contact or through contact with blood or infected toys [224, 226 - 229]. Horizontal transmission can also occur through body fluids such as semen and vaginal secretions [223, 224, 230].

Perinatal or vertical transmission of HBV occurs through blood or secretions from an infected mother to the newborn baby during delivery [225, 229, 231, 232]. Perinatal transmission is high in mothers who are hepatitis B e antigen (HBeAg) positive at 85 – 90 % and lower in those who are negative for HBeAg where the rate is 5 – 20 % [231, 233 - 235].

3.3.2 Global Epidemic of HBV Infection

Worldwide, it is estimated that more than 350 million people are currently living with chronic HBV infection, contributing to more than one million deaths annually [208, 236]. The prevalence of HBV infection is determined by the seroprevalence of HBsAg [237]. HBV is highly endemic in Asia and sub-Saharan Africa with HBsAg seroprevalence rates exceeding 8% (see **Figure 1.6**).

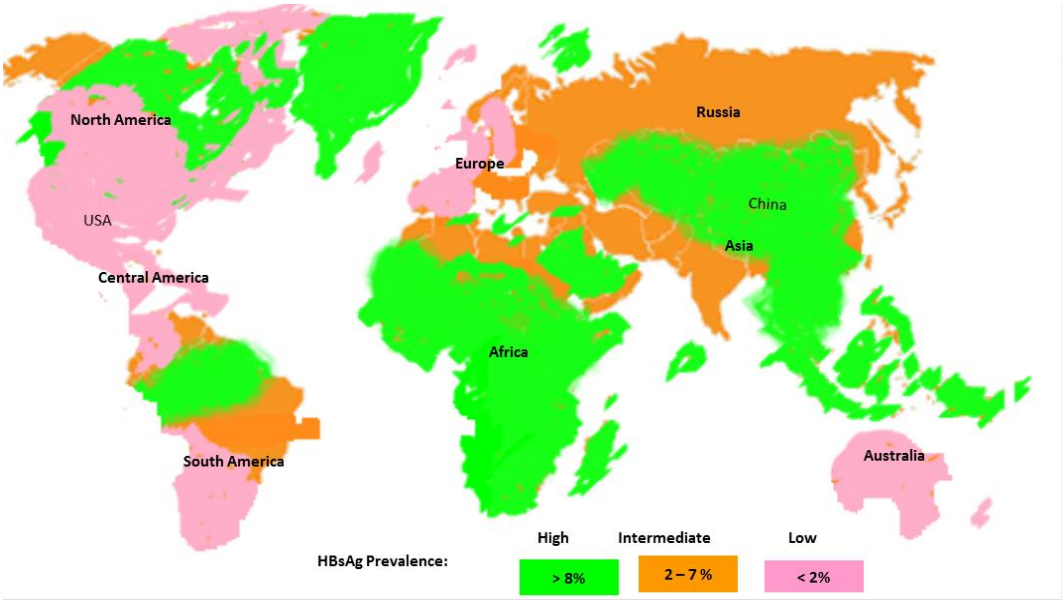

Figure 1.6: Global Geographical Distribution of Chronic Hepatitis B Infection (Adapted from Stannard[407]).

Abbreviations: **HBsAg** - Hepatitis B surface antigen, **USA** - United States of America.

In these regions, the infection is typically acquired at birth or in early childhood [207]. Progression to chronic HBV infection is common in these regions and is associated with prevalence rates of 30% for hepatic cirrhosis and 53% for HCC [209, 238, 239] .

Annually, approximately one million people are diagnosed with HCC worldwide and more than half of these people die within a year of diagnosis. Studies show that the highest HCC incidence rates of 70 – 80 % occur in Southeast Asia and sub-Saharan Africa, the regions with a high prevalence of chronic HBV infection [211, 212]. This is due to various factors that include the late presentation of patients with large tumours, failure to recognise those at risk, high prevalence of risk factors in the

population, lack of medical facilities for early diagnosis and limited access to effective treatment after diagnosis [212].

An intermediate HBsAg seroprevalence of 2 – 7 % is seen in some parts of Asia, Europe, America and Russia. The prevalence of HBV infection is low in Western Europe, Australia and United States where HBsAg seroprevalence is less than two percent [240].

3.3.3 Epidemic of HBV Infection in Africa

There are 65 million individuals infected with chronic HBV in Africa and 250 000 of these people die annually due to HBV related diseases. The prevalence of chronic HBV infection in Africa varies by geographic region. It is high in sub-Saharan Africa, with HBsAg seroprevalence rates of more than 8%. In Kenya, Sierra Leone, Zambia, Senegal and Liberia, the prevalence of HBV infection is intermediate with HBsAg seroprevalence rates ranging from 2% to 8%. North African countries including Morocco, Egypt, Algeria and Tunisia have low prevalence rates of less than 2% [241, 242].

In South Africa and other African countries, the prevalence of HBV infection is much higher in rural compared to urban areas [230, 243]. Low socio-economic status, infected household contact, unsafe sexual intercourse, sharing of partially eaten sweets or chewing gum, dental work and bathing towels may be some of the contributing factors for the high prevalence of HBV infection in rural areas [223, 229, 241, 244].

3.3.4 HBV Genotypes, Mutations and Genomic DNA Methylation

HBV is classified into eight genotypes (A - H) with four major serotypes (adw, adr, ayw and ayr) [245 - 249]. HBV genotypes are differentiated by more than 8% sequence divergence in the entire genome and more than 4% at the level of S gene [250, 251]. They have distinct geographical distribution as illustrated in **Table 1.3**. Genotype A is predominant in sub-Saharan Africa, north-west Europe and North America [252 - 254].

Table 1.3: The Global Geographic Distribution of HBV Genotypes, Mutations and Associated CpG Promoter DNA Methylation [245, 248 - 252, 255 - 258]

Genotype	Geographic Distribution	Mutation	Host CpG Promoter Methylation
A	North America, sub-Saharan Africa, north-west Europe	G1888A 1762T1764A G1862T	Induces hypomethylation and down-regulation of *DLEC1* gene
B	Indonesia, China, Vietnam	Unknown	Unknown
C	East Asia, Korea, China, Japan, Polynesia, Vietnam	Unknown	Unknown
D	Mediterranean area, Middle East	G1896A	Induces hypermethylation and down-regulation of *GSTP1* gene
E	Africa	Unknown	Unknown
F	Central and South America, Polynesia	Unknown	Unknown
G	France, America	Unknown	Unknown
H	Central America, California, Mexico	Unknown	Unknown

Abbreviations: A - Adenine , **CpG** - Cytosine-phosphate-Guanine, **DLEC1** - Deleted in Lung and Esophageal cancer 1, **G** - Guanine, **GSTP1** - Glutathione S transferase pi 1, **HBV** - Hepatitis B virus, **T** - Thymine.

Genotype A has four subgenotypes. Subgenotype 1A is common in South Africa, Malawi, Tanzania, Uganda, Somalia, Yemen, India, Nepal, Brazil and the Philippines. There are three CpG islands within HBV genotype A, which are associated with methylation of the promoter of *Deleted in Lung and Esophageal Cancer 1* (*DLEC*) gene and down-regulation of its expression in HBV-induced HCC [255, 259]. *DLEC* is a tumour suppressor gene and has been reported to be down-regulated in ovarian, liver, lung and EBV-related cancers [116, 255, 259].

Genotypes B and C are more prevalent in Asia, Indonesia and Vietnam [236, 247, 261, 445]. Genotype D is commonly found in the Mediterranean region and Middle East [252 -254]. The hepatitis B x (HBx) protein is associated with hypermethylation and down-regulation of the *GSTP1* gene which plays an important role in the development of cancer. Genotype E is found mainly in Africa. Genotype F is found in Europe and the United States, and genotype G in France and America [249, 251]**.** Genotype H is predominant in Central America, California and Mexico [258].

HBV has a mutation rate of 10%, which is relatively high compared to other viruses. It replicates via reverse transcription of RNA intermediates that result in random mismatched base errors during genomic replication. HBV DNA polymerase lacks the ability to proofread these errors and this predisposes HBV to mutations [262, 273]. HBV develops four major mutations which are the precore, basic core promoter, tyrosine-

methionine-aspartate-aspartate (YMDD) and asparagines-to-threonine (rtN236T) mutations. The precore mutants were the first to be identified and are characterised by a nonsense G1896A mutation [264, 265]. The G1896A mutation is responsible for HBeAg negativity in chronic HBV carriers and induces the down-regulation of HLA class II molecules in hepatocytes [266]. This mutation is common in individuals infected with HBV genotype D [234, 267]. The basic core promoter mutations include A1762T and G1764A and were identified after the precore mutations [235, 265]. Similar to the precore mutations, the basic core promoter mutations are found in HBeAg-negative individuals where they prevent HBeAg expression [265, 268].

3.3.5 Prevention and Treatment

HBV infection can be prevented by avoiding direct contact with any HBV contaminated fluids and materials. Immunization with recombinant hepatitis B vaccines is recommended for all infants at birth [416] and in individuals who are at high risk of acquiring the infection. Passive immunoprophylaxis with hepatitis B immunoglobulin derived from sera of positive HBV individuals is used to prevent mother-to-child HBV transmission at birth, after liver transplantation for HBV infection, needle-stick injuries and sexual intercourse [247, 417, 418].

Acute HBV infection does not require treatment as it usually resolves spontaneously [205, 206]. Two major classes of drugs available for treating chronic HBV infection include the injectable standard interferon-α and pegylated interferon-α2, and the oral nucleos(t)ide analogues. Nucleoside analogues are lamivudine, entecavir telbivudine, while nucleotide analoques are adefovir dipivoxil and tenofovir [343, 440, 419 – 425]. The main aims of treatment are to improve long-term survival by reducing the risk of developing cirrhosis and HCC [421, 423].

Treatment with oral nucleos(t)ide analogues is associated with the development of mutations [269, 270]. Lamivudine induces point mutations in the YMDD motif of the HBV polymerase and these include rtM204V and rtM204I mutations [271, 272]. The viral replication rate increases in the presence of lamivudine resistance and when lamivudine treatment is stopped, the wild-type virus re-establishes itself [272, 273]. Lamuvidine resistance mutations are responsible for the development of resistance in

entecavir that is also associated with similar mutations and more including rtI169T, rtT184G, rtS202I and rtM250V [118, 127, 260]. Telbivudine has a high antiviral potency and relatively low resistance than lamuvidine and entecavir. It is associated with mutations at rtL80I/V, rtL180M, rtA181T/V, rtM204I and rtL229W/V [441]. Telbivudine results in myoparthy and neuropathy when used simultaneously with pegylated interferon-α2 and therefore combination of these two agents is avoided [120].

Adevovir treatment causes mutations that are associated with the emergence of resistant strains such as the rtN236T mutation which is downstream to the YMDD motif [269, 270]. The use of adevovir treatment is now rare as it is associated with severe kidney injury, which may be a consequence of mitochondrial DNA depletion and activity of multidrug resistance-associated protein 4 [438, 439].

Despite the availability of treatment for chronic HBV infection, many patients will develop cancer and this remains a major medical problem worldwide. This may be attributed to HCC-associated risk factors such as the HBV genotype, alanine aminotransferase (ALT), HBV load and HBV surface antigen level, which may influence the response to chronic HBV treatment [426, 430, 431]. The response to interferon is significantly higher in patients infected with HBV genotype A compared to D and in patients with lower levels of HBV DNA and higher levels of ALT [357, 426 – 431, 435].

Aberrant methylation of promoter CpG islands is the primary epigenetic change seen during the course of HBV infection as it progresses to cirrhosis and HCC [207]. Such methylation is detected at higher rates in HCC tissues compared to liver cirrhosis without cancer [207, 221, 222]. In a recent large cohort study report by Tseng et al, high HBV surface antigen levels are associated with a risk of developing HCC even in the presence of low HBV DNA levels [435]. This finding may be due to a higher degree of viral HBV surface antigen integration into the host genome that would result in mutations and epigenetic alteration particularly DNA methylation, causing chronic liver damage, malignant transformation and HCC [432, 433, 435 - 437].

The association of DNA methylation with chronic HBV treatment was first observed during telbivudine treatment [434]. Telbuvidine is a thymidine agent that interacts with protein kinases to form telbivudine 5'–triphosphate via phosphorylation. Telbivudine

5'–triphosphate competes with thymidine 5'–triphosphate, leading to the suppression of HBV DNA polymerase and reduced viral replication [434]. Interestingly, telbuvidine was recently reported to correct HBV-induced histone methylation in HBV infected hepatocytes [434].

3.3.6 HBV Virion Structure, Genomic Organisation and Functions

HBV virions are infectious double-shelled particles of approximately 40 to 42 nanometre (nm) in diameter (see **Figure 1.7**). They consist of a nucleocapsid core of 27 nm in diameter, which forms the inner part of enveloped virions known as Dane particles [274]. The nucleocapsid core is surrounded by an outer surface antigen coat of ~4 nm thickness. It contains HBsAg and hepatitis B core antigen (HBcAg), which are detected in the sera of HBV-infected individuals in the form of spherical and filamentous particles [274 -277].

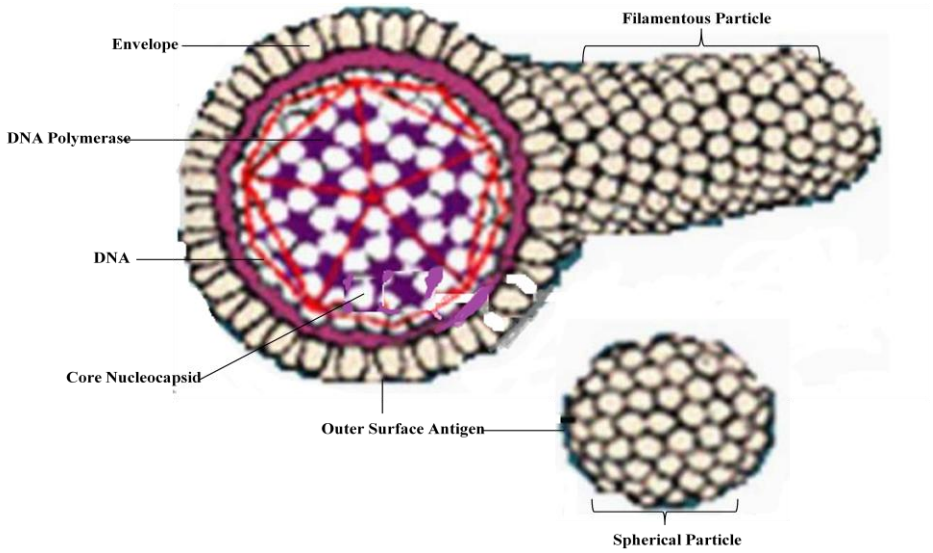

Figure 1.7: An Electron Micrograph Image of Hepatitis B Virion and Surface Antigen Components (Adapted from Otto et al[408]).

HBV is circular and partially double-stranded DNA virus of 40 to 42 nm in diameter. It has a nucleocapsid core of 27 nm in diameter, surrounded by an outer coat containing hepatitis B surface antigen of nearly 4nm thick that can be in the form of spherical and filamentous particles in the blood of HBV infected individuals.

Abbreviation: **DNA** - Deoxyribonucleic acid, **HBV**- Hepatitis B virus, **nm** - Nanometre.

HBV genome is a small and relaxed circular molecule of 3.2 kb in size (see **Figure 1.8**). It contains two strands of different length, a long minus strand and a short plus strand [276]. The minus strand is terminally redundant and contains a second copy of direct repeat 1 (DR1), ε signal and poly A tail. It serves as a template for reverse transcription of a plus strand and also as a transcript for the translation of viral proteins including polymerase, HBcAg and HBeAg [267]. The 5′ end of a minus strand is covalently linked to the viral reverse transcriptase and polymerase through a phosphor-tyrosine bond. The plus strand overlaps part of the minus strand whilst its 5′ end bears the oligoribonucleotides [276].

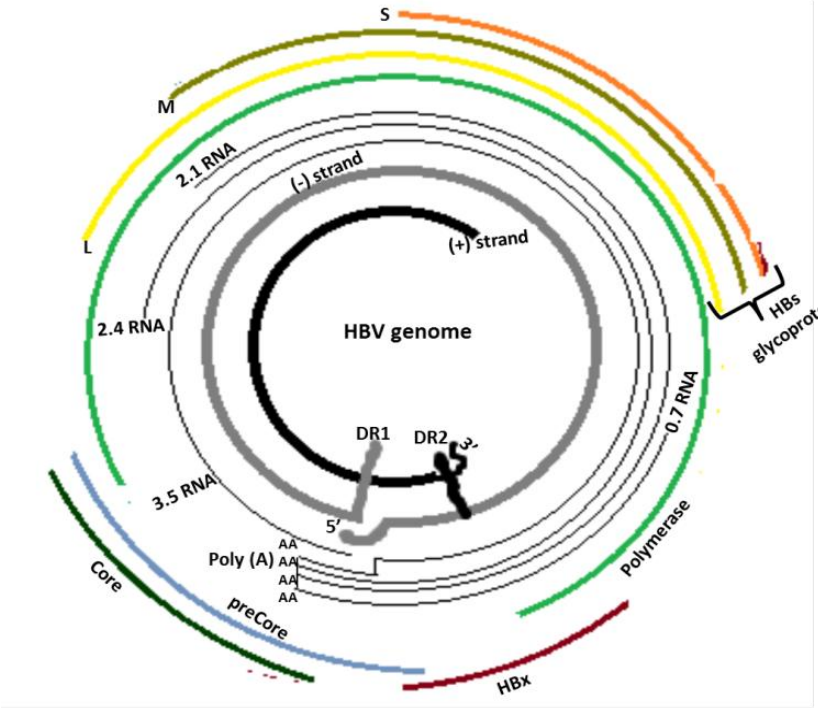

Figure 1.8: The Structure of the HBV Genome (Adapted from Ganem and Prince[206]).

The inner circles represent two DNA strands, the full-length minus and the incomplete plus strands. The thin black lines represent four viral RNA transcripts of different length. The outermost coloured lines indicate HBV surface glycoproteins (Large, Middle and Small), Polymerase, HBx and precore/core proteins.

Abbreviations: (-) - Negative, (+) - Positive, **DR** - Direct repeat, **HBs** - Hepatitis B surface, **HBx** - Hepatitis B x antigen, **HBV** - Hepatitis B virus, **L** - Large, **M** - Middle, **Poly (A)** - Polyadenylation, **RNA** - Ribonucleic acid, **S** - Small.

The HBV genome contains four ORFs, which have the same orientation and partially overlap [278]. These ORFs encode the viral envelope pre-S/S, a pre-core/core, a polymerase and X proteins. The viral envelope also encodes three surface glycoproteins, which are the Large (L), Middle (M), and Small (S) glycoproteins [279, 280]. These surface glycoproteins are synthesised by the initial transcription of pre-S/S. The L surface glycoprotein is important for viral assembly and infectivity, while the function of M surface glycoprotein is unknown [281].

The longest open reading frame encodes the viral polymerase which serves as a reverse transcriptase and DNA polymerase [282]. The pre-S/S envelope open reading frame overlaps the precore/core and X open reading frames, and encodes HBsAg [208]. The precore/core open reading frame produces HBeAg and HBcAg through cleavage by cellular proteases. HBcAg is the nucleocapsid and encloses the viral DNA [267, 278].

HBx protein is a transactivating protein that alters the expression of some genes via DNA methylation leading to tumorigenesis [283 - 286]. It consists of 154 amino acid residues with a molecular weight of 27kD and is encoded by the smallest ORF [287]. It stimulates viral replication either by activating viral transcription or by enhancing the reverse transcription of the viral polymerase [213, 283, 288]. In hepatoma cell lines, HBx protein enhances viral replication by interacting with DNA binding protein 1 which interferes with cell growth and viability. In mice infected with wild-type HBV, viral replication is stimulated by HBx protein, suggesting that HBx protein is required for viral replication in normal hepatocyte cells [287, 289].

3.3.7 Life-Cycle of HBV

Due to the lack of efficient *in vitro* infection systems and animal models in which to study the life cycle of HBV infection, a lot of data is from the duck model infected with duck hepatitis B virus (DHBV) [277, 290]. HBV life-cycle begins through the interaction of HBsAg with cellular receptor/s at the surface of hepatocytes (see **Figure 1.9**).

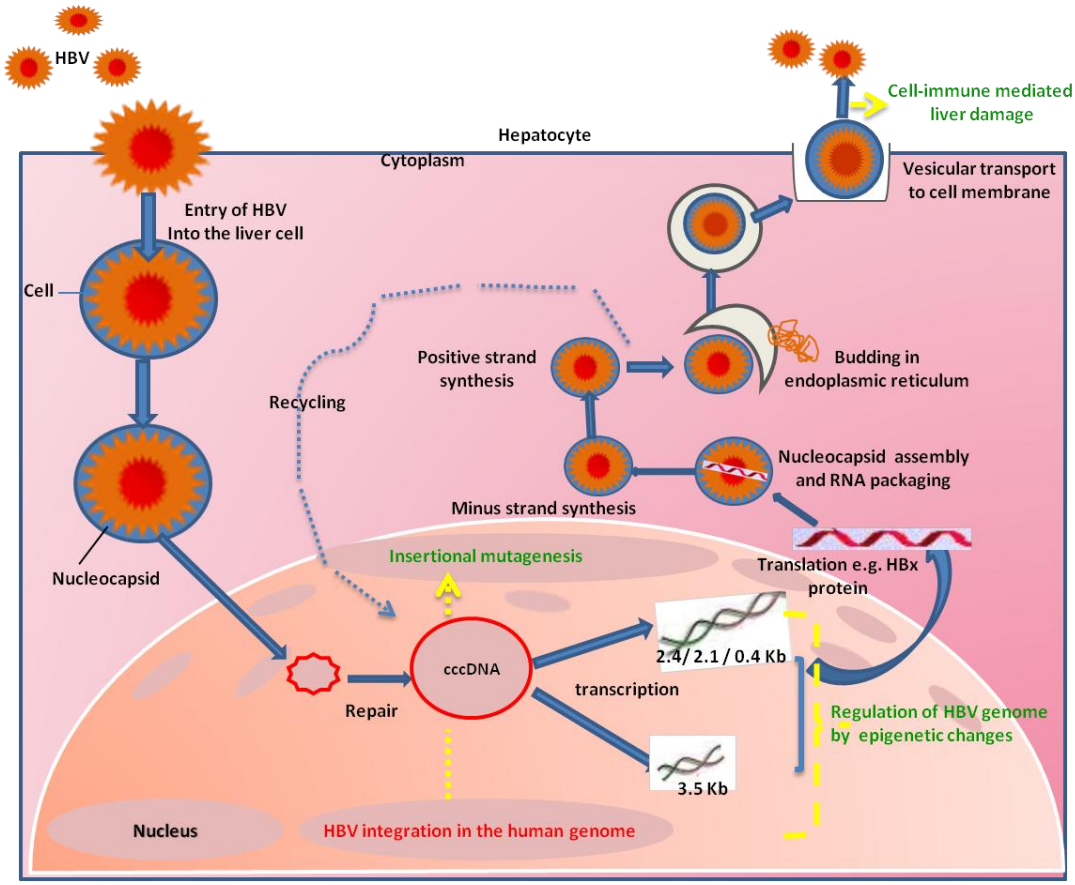

Figure 1.9: The Life-Cycle of HBV Infection (Adapted from Ganem and Prince[206]).

During the entry of HBV into hepatocytes, the genome moves to the nucleus where is converted into cccDNA. The cccDNA is transcribed and moves to the cytoplasm. The viral polymerase uses reverse transcription to form pregenomic RNA and viral transcripts, which are then translated to form viral proteins. Molecular mechanisms that occur during HBV infection and may explain the transition of chronic HBV infection to HCC are shown in green colour and highlighted with yellow arrows.

Abbreviations: **cccDNA** - Covalently closed circular deoxyribonucleic acid, **HBV** - Hepatitis B virus, HBx - Hepatitis B x protein, **HCC** - Hepatocellular carcinoma, **RNA** - Ribonucleic acid.

A number of potential cellular receptors that interact with HBsAg during HBV infection have been previously identified, but the mechanisms of action still remain controversial as none of them has been proved to be functional to HBV. These receptors include retinoid X receptor (RXR), peroxisome proliferator-activated receptor (PPAR) and farnesoid X receptor (FXR) [291, 292].

Recently, sodium taurocholate cotransporting polypeptide (NTCP) was discovered as the potential receptor for HBV infection [414]. NTCP is abundantly expressed in the

liver and is involved in the transportation and clearance of bile acids from portal blood into hepatocytes [415]. Yan et al[414] have shown by using near zero distance photo-cross-linking, tandem affinity purification and mass spectrophotometry that the pre-S/S envelope domain, a key determinant for receptor/s binding, selectively interacts with NTCP to facilitate HBV infection. Knockdown of the NTCP expression in duck primary hepatocytes infected with DHBV significantly decreased HBV infection, suggesting that NTCP is actually required for HBV infection [414].

HBV requires DNA polymerase and reverse transcriptase to replicate through RNA intermediates known as pregenomic RNA [293]. Following the interaction of surfaces antigen with cellular receptor, the viral nucleocapsid enters the host cell's nucleus to deliver dsDNA. In the nucleus, the dsDNA gets repaired and converted to covalently closed circular super-coiled DNA (cccDNA) by DNA polymerase [263, 294]. The cccDNA molecule serves as a template for the transcription of four viral RNA transcripts 3.5, 2.4, 2.1 and 0.4 kb in size, pregenomic RNA and RNA intermediate for viral replication before moving to the cytoplasm [293, 295, 296]. The mRNA transcripts are then translated to produce the envelope (pre-S/S), precore/core, viral polymerase and X proteins. The 3.5 RNA transcript is reverse-transcribed into viral dsDNA [293, 294]. Some of the resulting viral DNA and polymerase containing capsids are enveloped via budding into the endoplasmic reticulum (ER). The rest of the viral DNA is recycled or is migrated back to the nucleus where it produces new generations of cccDNA which maintains persistent HBV infection [297].

4. HBV-INDUCED HCC AND DNA METHYLATION

Three CpG islands are identified in episomal HBV DNA. These are island 1 located on nucleotide positions 55-286, island 2 on 1224-1667 and island 3 on 2257-2443 [298, 299]. Methylation of CpG islands in the human genome is known to regulate gene transcription. This prompted Vivekanandan et al [300] to hypothesise that methylation of CpG islands in HBV DNA may regulate viral gene expression. To test this hypothesis, *in vitro* methylation of the transfected HBV DNA was done and this resulted in decreased expression of HBV mRNA and proteins in the cells. In addition, the effect of viral cccDNA methylation in the liver tissue of patients with

chronic HBV infection was investigated and found to be associated with reduced HBV replication [300]. These findings support the work of Pollicino et al [301] who showed that HBV replication is regulated by the acetylation of HBV cccDNA bound H3 and H4 histone proteins. Although, this data suggest that HBV DNA methylation is a novel mechanism that influences the regulation of viral gene expression, the mechanisms of action are still not known.

Previous human studies have shown that DNA viruses integrate into the host genome and that the expression levels of DNMTs increase in response to active viral replication [136, 140, 141,191, 302, 303]. Vivekanandan et al [303] hypothesised that the upregulation of DNMTs give infected cells the ability to methylate viral DNA and therefore control viral replication. To investigate this, the expression of DNMTs was measured in cell lines exposed to HBV DNA using two experimental systems, one of temporary transfection of cells and another that mimicked natural chronic infection. High levels expression of DNMT1, 2 and 3 were observed in response to persistent HBV infection. This correlated with suppressed viral replication associated with methylation of HBV DNA and increased methylation of host CpG islands [303].

The seminal work of Vivekanandan et al[303] allows for the development of a model that explains the development of liver injury and HCC in chronic HBV infection (see **Figure 1.10**). In this model, infected host cells respond to HBV infection by upregulating the expression of DNMTs. Upregulation of DNMTs can also result from interaction with HBx transcriptional activator protein [303]. Once activated, DNMTs methylate HBV DNA and switch off the expression of viral mRNA and proteins, thereby reducing viral replication [303]. The methylation of integrated HBV DNA may be detrimental to the host genome through the inappropriate methylation of the neighbouring host genome, particularly if the promoter CpG islands regions of the gene are affected. A consequence of this effect would be the transcriptional repression of host immunoregulatory and tumour suppressor genes that prevent the development of cancer [303].

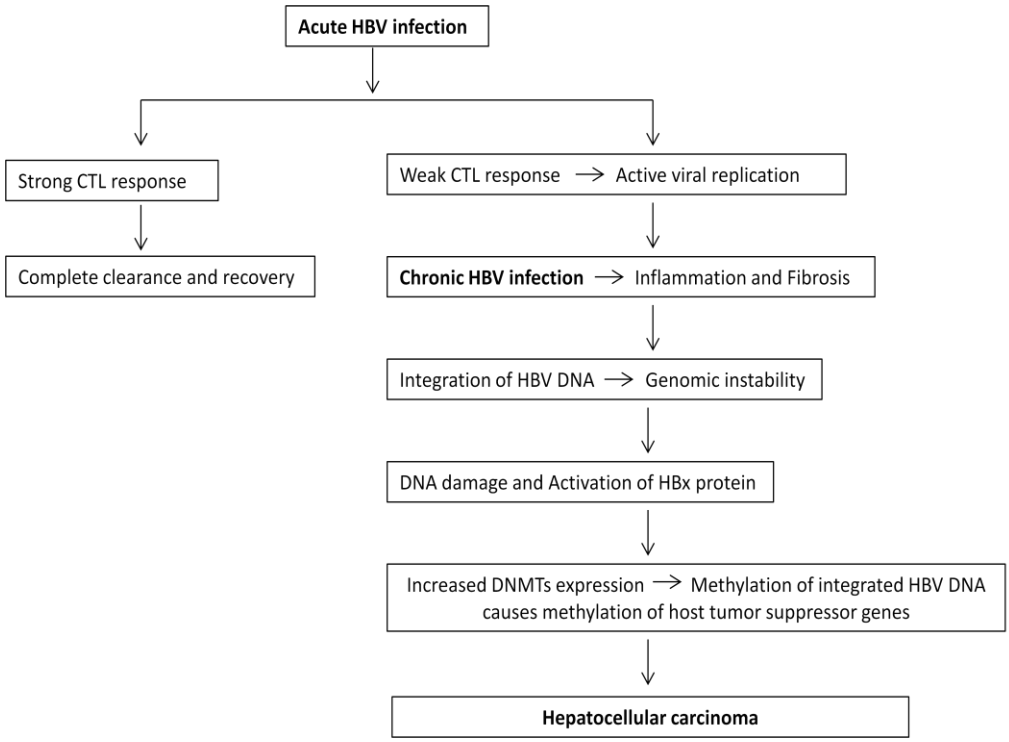

Figure 1.10: Model of Chronic HBV Infection and DNA Methylation.

HBV integrates into the host genome and promotes viral persistence. Infected cells increase the expression of DNMTs in response to viral replication. This causes methylation of HBV cccDNA and reduces viral replication. The same methylation system methylates the adjacent host tumour suppressor and immunoregulatory genes leading to hepatocarcinogenesis.

Abbreviations: **cccDNA** - Covalently closed circular deoxyribonucleic acid, **CTL** - $CD8^+$ cytotoxic T lymphocytes, **HBV** - Hepatitis B virus, **HBx** - Hepatitis B x protein.

4.1 Adaptive Immune Responses and Liver Damage

During acute HBV infection, some individuals successfully clear the virus without any evidence of liver disease [304 - 306] (see **Figure 1.11**). However, HBV can be detected in blood several years after clinical recovery from an acute infection [307]. The continuous presence of HBV DNA stimulates an active immune response that over time can cause liver damage [216, 308 - 310]. The chronic inflammatory and regenerative processes are susceptible to develop mutagenic changes [311, 312]. In turn, mutagenesis damages the DNA thereby increasing the chances of malignant transformation [313, 314].

The $CD4^+$ cells are important component in the adaptive immune response in acute HBV infection [311, 315 - 317]. When activated, they produce $CD8^+$ cytotoxic T-

lymphocytes (CTL) that helps in controlling and eliminating virus by killing infected cells [318, 319]. HBV specific CTL responses are stronger in acute compared to chronic HBV infection [320]. They can persist for decades following viral clearance and recovery from acute HBV infection (see **Figure 1.11**).

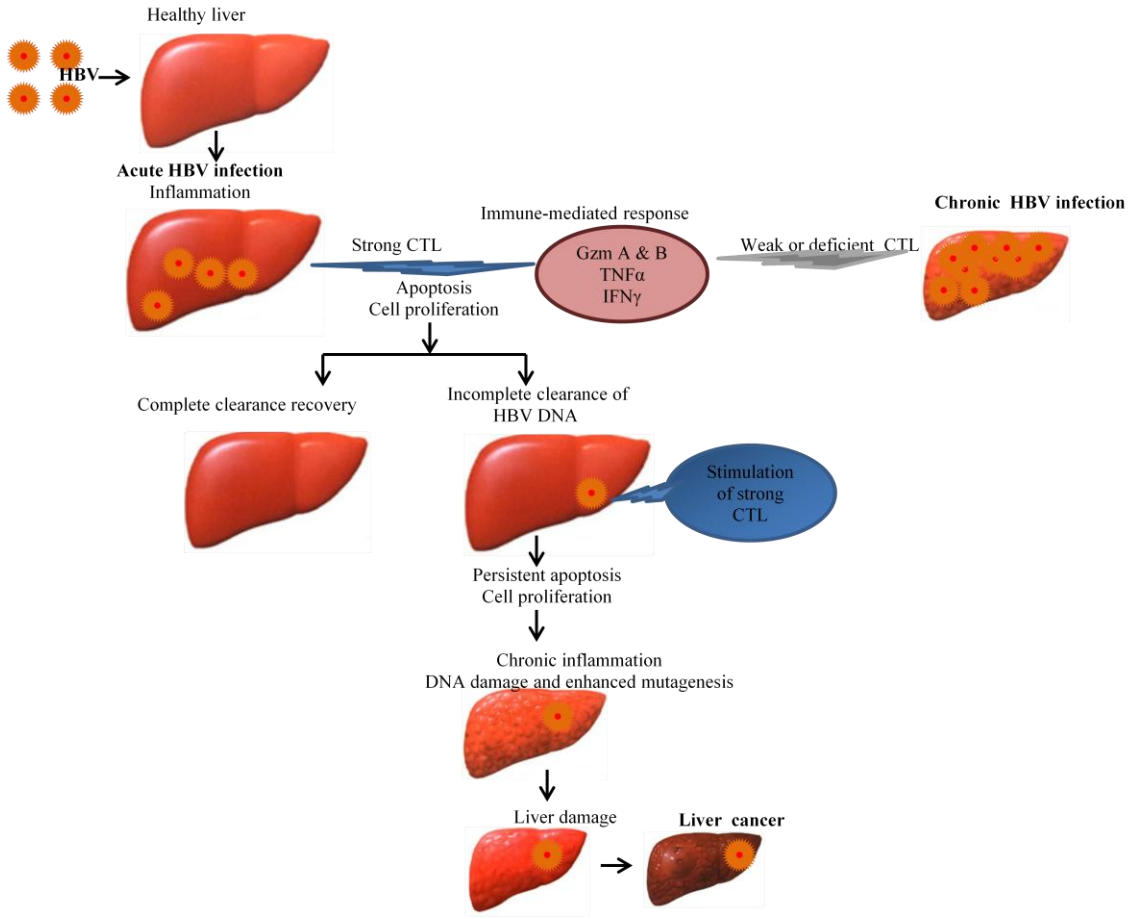

Figure 1.11: Adaptive Immune Response and Liver Damage in Chronic HBV Infection.

In acute HBV infection, individuals with strong immune responses clear the virus. Patients with weak immune responses fail to clear the virus and develop chronic HBV infection.

Abbreviations: **HBV** - Hepatitis B virus, **HCC** - Hepatocellular carcinoma, **IFNγ** - Interferon gamma, **TNFα** - Tumour necrosis factor alpha.

During interaction with infected hepatocytes, CTL activate the caspase-3 dependent pathway and express serine protease granzymes A and B [321, 322]. Granzymes A and B induce apoptosis and inflammation via interleukin-1β [323 - 325]. They are controlled by

perforin molecules and increase reactive oxygen species (ROS) by cleaving mitochondrial complex I protein and nicotinamide adenine dinucleotides hydrate dehydrogenase (NADH) Fe-S protein 3 [326 - 328]. This causes single- or double-stranded nicks in the DNA thereby killing HBV-infected cells [329, 330].

Serine protease inhibitor Kazal (SPIK) is an inflammatory protein that is abundantly expressed in response to high HBV and hepatitis C virus (HCV) replication [331, 332]. In chronic HBV infection, activation of SPIK causes uncontrolled HBV replication by inhibiting granule enzyme A mediated apoptosis. High level of SPIK expression suppresses serine protease granzymes-dependent cell apoptosis, resulting in the induction of epigenetic alterations that can cause HBV-induced HCC [325, 333, 334].

In chronic HBV infection, the HBV DNA enhances immune responses cause apoptosis and cell proliferation and may lead to hepatocarcinogenesis [335, 336]. The CTL responses are robust, polyclonal, multispecific and target HBV-encoded antigens. Studies show that during chronic HBV infection with active viral replication and weak or pharmacologically suppressed CTL, there is minimal liver damage or injury [337, 338]. HBV is therefore noncytopathic and damage to the liver is due to activation of CTL responses that keep viral replication under control [319].

Cytokines such as tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ) inhibit HBV replication and activate macrophages that cause liver damage and contribute to hepatic inflammation [314, 335]. In a HBV transgenic mouse model, TNFα and IFNγ eliminate the circulating virus in a proteasome- and kinase-dependent manner [313, 316]. Persistent production of TNFα and IFNγ in response to viral replication may cause genetic mutations that lead to HCC in *in vivo* models of HBV [310, 314, 335, 339].

Individuals who fail to eradicate the virus due to deficient or weak immune responses develop chronic HBV infection that is often associated with active viral replication and minimal liver injury [306, 340] (see **Figure 1.11**). Weak or deficient immune responses are thought to be due to poor CTL production, deletion or exhaustion [306, 341, 342]. Such patients are more likely to remain asymptomatic with little progression of their liver disease. However, about 10 – 30 % may develop liver cirrhosis with the risk of liver cancer [306, 343].

4.2 Integration of HBV DNA into the Human Genome

Deficient CTL immune responses lead to chronic HBV infection which enables viral integration into the host genome and epigenetic alteration of host tumour suppressor genes. These events may lead to the development of HBV-induced HCC [303].

HBV integration was first discovered in 1980 using Southern blot hybridization. It was associated with genomic instability such as loss of heterozygocity (LOH), resulting in the rearrangements, deletions, duplications and inversions of the host and viral genomic sequences [210]. Viral integration results in the insertion of HBV DNA sequences such as HBx gene in the host genome and enables viral persistence [344].

Integration of HBV in the host genome also occurs in woodchucks and other animal models [344 - 346]. In woodchucks and California ground squirrels (Spermophilus beecheyi), HBV genome integrates close to *ras* and *myc* family oncogenes including *c-myc*, *N-myc1* and *N-myc2* [347 - 349]. Modulation of *myc* and *ras* family oncogenes through *cis*-activation enhances cell proliferation and transformation. These events occur via transactivation action of HBx protein and favour the development of cancer [350].

The occurrence of integrated HBV DNA at preferential sites in the human chromosomes has been identified using Alu-PCR based-technique. The preferential sites are known as chromosomal fragile sites (CFS) and are non-random [344]. HBV DNA integrates into the human genome soon after the repair and conversion of HBV DNA to cccDNA [199, 298, 300, 351]. The HBV genome integrates within the coding sequence or close to an array of key regulatory cellular genes that can deregulate proto-oncogenes and tumour suppressor genes. Activation or inactivation of such genes promotes genomic chromosomal instability by altering various cellular signalling pathways, triggering genetic mutations and epigenetic alteration [344, 352, 353].

Mutagenesis and epigenetic alteration result in the abnormal regulation of the targeted genes. This promotes malignant transformation by altering the control of cell growth, differentiation, proliferation and apoptosis [344, 351, 354, 355]. The integration of HBV at or within *cyclin A* and *RARβ* genes is associated with increased protein activities and hepatocellular growth in HBV-induced HCC, suggesting that HBV integration contributes to hepatocytes transformation [350]. Examples of known active

CFS targeted by HBV integration are outlined in **Table 1.4**. The 60s Ribosomal protein, *hTERT*, *major histocompatibility complex I like leukocyte* (*Mill*), *platelet derived growth factor receptor (PDGFR)* and *calcium signalling related* genes are also common sites or targets of HBV integration. These genes are important in cellular signalling pathways that control DNA damage, oxidation stress and cell growth, and their alteration is associated with development and progression of cancer [350, 356, 357].

Table 1.4: Examples of Chromosomal Fragile Sites Associated with HBV Insertions and Their Roles in Tumour Development [344, 350, 356, 357]

Chromosomal Fragile Sites	Target Gene	Role in Tumor Development
FRA1A (1p36)	TCEA; RAR; CHML	Alters gene expression and Promote cell survival
FRA2C (1q)	EMX2-like gene	Modulates β-catenin signaling pathway and cell survival
FRA4E (4p)	Cyclin A	Stimulate cell-cycle and anti-apoptotic effect
FRA3D (3q25.3)	IRAK2	Promotes Apoptosis and tumor progression
FRA5C (5p31.1)	PDGFRβ	Regulates DNA synthesis and fibrotic genes
FRA7 (7p)	SERCA 1; NCF1	β-catenin activation
FRA9 (9q)	KLF1; CASPR3	Promote cell growth; Regulates DNA methylation
FRA10A (10q)	PTEN; PI3K	Promotes metastasis
FRA11A (11q13)	EMS1, FGF4; BIRC3	Promotes cell cycle progression
FRA12A (12q24)	ErbB3; Mill2	Modulates β-catenin signaling pathway; Alters cell fate
FRA13A (13q32)	CTGF; CCNL; IMP-2	Promotes tumor progression
FRA18 (18q)	DCC; DPC4	Tumor suppression
FRA19A (19q13)	Cyclin E	Regulates methyl-CpG- binding proteins
FRA20 (20P12.3)	hTERT	Delay DNA synthesis; Promotes immortalization

Abbreviations: **BIRC3** - Baculoviral IAP repeat containing 3, **CASPR3** - Contactin-associated protein-like 3, **CCNL** - Cyclin L1, **CHML** - Choroideremia-like gene, **CTGF** - Connective tissue growth factor, **DCC** - Deleted in colorectal cancer, **DPC4** - Deleted in pancreatic cancer 4, **EMSL** - EMSL, **EMX2** - Empty spiracle homeobox 2, **ErbB3** - V-erb-b2 erythroblastic leukemia viral oncogene homolog 3, **FGF4** - Fibroblast growth factor 4, **FRA** - Fragile site, **hTERT** - Human telomerase reverse transcriptase, **IMP-2** - Insulin-like growth factor II mRNA binding protein 2, **IRAK2** - Interleukin-1 receptor associated kinase 2, **KLF1** - Krueppel-like factor 1, **Mill2** - Major histocompatibility complex I like leukocyte 2, **NCF1** - Neutrophil cytosolic factor 1, **PDGFRβ** - Platelet derived growth factor receptor beta, **PI3K** - Phosphatidylinositol 3 kinase, **PTEN** - Phosphatise and tension homolog, **RAR** - Retinoic acid receptor, **SERCA** - Sarco/endoplasmic reticulum calcium transport ATPase, **TCEA** - Transcription elongation factor A.

4.3 Hepatitis B Virus X Gene or Protein and Its Carcinogenic Effects

HBx protein is a transcriptional transactivator that HBV uses to integrate into the host cellular DNA and is associated with malignant transformation in hepatocytes [288, 289, 357 - 359]. It interacts with nuclear transcription factors such as NF-κB, AP1, CREB, TATA-binding protein (TBP), peroxisome proliferator-activated receptor γ (PPARγ) and transcription factor II H (TFIIH) [360, 406]. Interaction of HBx protein with these transcription factors disrupts multiple cellular signalling pathways that include janus kinase 1 (JAK1)-signal transducer activator of transcription (STAT), mitogen-

activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and p53 signalling pathways [361 - 364]. Cellular signalling pathways are important in regulating DNA repair, cell growth, differentiation, adhesion, proliferation and apoptosis [359, 362, 365 -367]. Although the precise mechanisms of action are still being elucidated, HBx protein has also been shown to induce methylation of important tumour suppressor genes critical in HBV-induced hepatocarcinogenesis by modulating DNMTs [289, 368, 369].

4.3.1 HBx Protein as a Transcriptional Activator

The transcriptional transactivation role of HBx protein on the transforming growth factor beta 1 (TGF-β1) protein may be important in explaining liver inflammation and fibrosis [329, 370]. TGF-β1, encoded by *TGF-β1* gene, is a cytokine that is produced in response to liver injury by activated hepatocytes, platelets and Kupffer cells [371]. It triggers apoptosis, cell growth and differentiation in human hepatocytes, hepatoma cell lines and transgenic mice [152, 370, 372 - 374]. It promotes the development of fibrosis and cirrhosis in chronic HBV infection and other liver-related diseases [372, 375].

HBx protein induces the expression of TGF-β1 through the transactivation of *TGF-β1* gene, the down-regulation of α_2-macroglobulin and the induction of TGF-β1 mediator Smad4 [376, 377]. High levels of TGF-β1 protein are observed in the sera of chronic HBV-induced HCC patients and correlate with the mutation and loss of mannose-6-phosphate/IGF-II receptor that mediates TGF-β1 signalling [373, 378 - 379]. In addition, HBx protein alters the signalling pathway of TGF-β1 from being tumour suppressive to oncogenic in early chronic HBV infection. This occurs via the activation of c-Jun N-terminal kinase (JNK) which shifts epithelial tumour suppressive pSmad3C signal to mesenchymal oncogenic pSmadL signal pathway [380].

Studies show that in HBx transgenic mice and hepatoma cell lines, HBx protein can transactivate the NF-κB, MAPK/ERK, STAT3 and PI3K/ Akt cellular signalling pathways by inducing the production of ROS [329, 353, 381 - 383]. Accumulation of ROS in human cancers is associated with anti-apoptotic activity, DNA damage and mutations which promote malignant transformation [328, 329, 382]. HBx-induced ROS and 8-oxoguanine alter the expression of PTEN protein by oxidising cysteine residues

within the promoter region encoding *PTEN* gene, which activate Akt pathway and contributes to hepatocarcinogenesis [329, 365, 383].

4.3.2 HBx Protein, Proliferation and Apoptosis

Apoptosis is an important process in normal development and viruses like HBV that are associated with tumorigenesis [78]. Damage to cellular DNA induced by a variety of stimuli such as HBV infection and toxins often triggers localised apoptotic-related signals resulting in tissue necrosis. This process enables the elimination of damaged, unwanted and redundant hepatocytes that may otherwise lead to uncontrolled cell growth, proliferation and liver disease [79, 80]. Abnormal regulation of cell proliferation and apoptosis has been identified as the repercussion of inappropriate inactivation or activation of gene transcription in many diseases. These same processes are the central mechanisms which link chronic HBV infection to HCC development [8, 9].

HBx protein is mainly found in the cytoplasm, mitochondria and nucleus in which it has emerged as the central factor that stirs up the entire cellular signalling machinery to establish and maintain viral replication following infection both *in vivo* and *in vitro* [10 - 15]. It exerts its transcriptional transactivation activities by altering various functions of cellular genes and related signalling pathways [15]. Some of these pathways include p53, cyclooxygenase-2 (COX-2), myloid leukemia cell differentiation 1 (Mcl-1), phosphatydylinositol 3 kinase (PI3k) and NF-κB apoptotic-related pathways which when disrupted inhibits or induce abnormal apoptosis.

The tumour suppressor gene *p53* interacts with DNA and activates the transcription of reporter genes located on the downstream binding site of p53. It induces cell-cycle arrest in the G_1 phase and apoptosis in response to DNA damage. Normal regulation of apoptosis by p53 occurs through its interaction with cytoplasmic transcription factors. These transcription factors include ankyrin-repeat containing and proline-rich region-containing proteins (ASPP), fatty acid synthase, $p21^{CIPWARF1}$, xanthoma pigmentosa B (XPB) and repair cross-complementing rodent-repair deficiency group 2 (ERCC2). They are involved in the p53-mediated nucleotide excision repair and enhance the binding of *p53* gene to proapoptotic stimuli [15, 16, 385, 399, 402, 406].

HBx protein inhibits apoptosis by forming a complex with p53 protein and blocking its transcriptional transactivating properties and protein to protein interaction with transcription factors [358, 386]. For instance, HBx protein suppresses the p53-mediated DNA sequence-specific transcriptional activity by binding to its TFIIH promoter. This abolishes the role of p53 in nucleotide excision repair, cell cycle and apoptosis, resulting in an increased susceptibility of infected cells to p53 carcinogen-induced mutations [404, 405]. Inhibition of p53 protein-mediated apoptosis also occurs through the activation of COX-2-prostaglandin E_2 and McI-1 anti-apoptotic pathways.

HBx protein can also prevent apoptosis via the inhibition of caspase cascade 3 and the transactivation of NF-κB and PI3K signal transduction pathways but the mechanisms underlying these processes are still unknown [387 -389].

Induction of apoptosis was observed after interaction of HBV x-associated protein (XAP3) with protein kinase C (PKC) that switches on the transcription factor NF-κB through activation by HBx protein [75, 76]. HBx protein also induces apoptosis by reducing the cellular FLICE like inhibitory protein activity and increasing the activities of JNK, caspases 3 and 9 pro-apoptotic pathways which mediate apoptosis [409, 410]. Being localised in the cytoplasm also gives the HBx protein the ability to sensitize HBV infected cells towards these pathways and induce apoptosis [409].

4.3.3 HBx Protein and DNA Methylation

HBx protein has been labelled an epigenetic deregulating agent. It uses its oncogenic ability to induce promoter hypermethylation of some cellular tumour suppressor genes that contribute to the development of liver cancer [384]. By modulating the transcriptional activation of DNMTs, HBx protein induces the hypermethylation of tumour suppressor gene promoters and silences their expression [391,398,400, 401].

HBx protein induces the hypermethylation of *RARβ2* gene by upregulating DNMT1 and 3A activities and down-regulating the expression of RARβ2 protein [384, 390]. *RARβ2* binds to and inactivates the E2F1 transcription factor, which is essential for cell cycle progression [358, 391]. Downregulation of RARβ2 protein expression is associated with activation of E2F1 transcription factor, which abolishes the ability of retinoic acid to regulate expression of G_1 checkpoint regulators, leading to

upregulation of p16, p21 and p27 proteins. The activation of E2F1 transcription factor is associated with uncontrolled cell proliferation which contributes to carcinogenesis [390].

Insulin-like growth factor binding 3 (*IGJBP-3*) is another potential tumour suppressor gene which is both hyper- and hypomethylated in HBV induced HCC [289]. Hypermethylation of *IGJBP-3* gene is mediated by DNMT 1 and 3A which are upregulated via the transcriptional activities of HBx protein, and this is associated with loss of *IGJBP-3* gene expression. In contrast, HBx protein reduces the transcriptional activities of DNMT 3B, leading to hypomethylation and upregulation of the *IGJBP-3* gene [289].

DLEC1 is a functional tumour suppressor gene silenced by promoter methylation in lung, gastric, colon and nasopharyngeal cancers [116, 117, 255, 411, 412]. Similar methylation has also been observed in HCC where it is associated with induction of G1 cell cycle arrest and loss of gene expression [259]. Silencing of *DLEC 1* gene expression is mediated by both DNA hypermethylation and histone acetylation [411, 412]. HBx protein encoded by HBV genotype A enhances the transcription of *DLEC 1* gene by increasing the level of histone acetylation through activation of HATs, leading to suppression of tumour progression [255]. Through the activation of DNMT1 expression mediated by the pRB-E2F pathway, HBx protein induces DNA hypermethylation of *DLEC1* gene and suppresses its transcriptional activities [255].

Caveolin- 1, encoded by c*aveolin-1* gene, is an integral membrane protein abundantly expressed in adipose, fibrous and endothelial tissue [396]. High level expression of Caveolin-1 protein disrupts growth factor signalling pathways, which in turn alters cell growth, proliferation and differentiation [396, 413]. HCC cells expressing high levels of Caveolin-1 are associated with uncontrolled cell growth, motility, *in vivo* tumour aggressiveness and metastasis [413]. Conversely, HBx-induced methylation of *Caveolin-1* gene promoter region suppresses its transcriptional activities and this correlates with reduced tumour aggressiveness and metastasis, indicating a role of DNA methylation in HBV related HCC [396].

Hypermethylation of $p16^{ink4a}$ gene is a frequent event in several malignancies including HBV induced HCC [110, 116, 130, 391, 392, 395, 398]. HBx protein silences the expression of $p16^{ink4a}$ gene through the activation of DNA methyltransferase 1 and

the cyclin D1-CDK 4/6-pRb-E2F1 pathway [396]. Methylation of *p16*^{ink4a} gene is associated with increased viral replication, integration and loss of protein expression [398].

HBx protein induced DNA hypermethylation has also been connected with loss of expression and normal function of *LINE-1*, *pRB*, *ASPP*, *E-cadherin*, *GSTP1* and *hTERT* tumour suppressor genes [111, 113 - 116, 256, 302, 394, 397 - 399, 400 - 402]. This methylation is associated with increased upregulation of DNMTs with DNMT1being the most active one. Aberrant methylation of these genes is associated with perturbed cellular signalling pathways such as ubiquitination, DNA repair, transcription, proliferation and apoptosis, which may lead to the development of HBV related HCC [255, 259,289, 393.]

HCV is an RNA virus and unlike HBV, is unable to integrate into the human genome. However, it is able to cause epigenetic changes that favour its own replication and are associated with the development of liver cancer. Apolipoprotein E, which is required for the replication and infectivity of HCV is known to be hypermethylated in chronic HCV infection. This is associated with increased viral replication and an increased risk of developing malignancy[446 - 448].

5. SUMMARY

Studies show that there is an association between the methylation of CpG islands and transcriptional changes in gene promoter regions. Transcriptional alterations within gene promoter regions interfere with the normal function of a wide spectrum of cellular genes including tumour suppressor genes which are potential inducers of malignancies [41, 42, 48, 112].

Oncogenic viruses integrate themselves into the human genome and alter gene transcription through DNA methylation [137 - 139]. During HBV infection, the expression levels of DNMTs are elevated in response to viral replication as viral genes are methylated to suppress viral replication. This may result in inappropriate random methylation of neighbouring host cellular genes, including tumour suppressor genes. This would cause malignant transformation and ultimately liver cancer [303]. In addition, other genes affected by methylation may contribute to the development of liver inflammation, fibrosis and cirrhosis.

As a multifunctional viral transactivator, the HBx protein may be the driving force behind the activation of DNMTs, causing gene promoter hypermethylation and gene silencing. The epigenetic alteration of genes may affect cellular signalling pathways and favour uncontrolled hepatocyte proliferation and HBV-induced inflammation, fibrosis and cancer [288, 289, 362-370, 376, 377, 380, 384, 390 - 402].

Data on HBV induced DNA methylation has been obtained largely through the analysis of single genes using methods with variable levels of sensitivity to detect methylation such as bisulfite DNA sequencing and methylation specific PCR (MSP). These assays target only known methylated CpG promoter regions and therefore leave other potential CpG sites unidentified [403]. There is therefore value in investigating genome-wide methylation of promoter region CpG islands in patients with chronic HBV infection. Such studies may provide information that will contribute to the further understanding of HBV induced liver injury and cancer.

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CHAPTER 2

STUDY HYPOTHESIS, CLINICAL SETTING AND AIMS

2.1 HYPOTHESIS OF THE STUDY

The literature review discussed in **Chapter 1** demonstrated that methylation of HBV DNA integrated into the host genome is associated with transcriptional alteration and silencing of host genes. These changes result in several biological effects including the development of cancer [1 - 5].

Given these observations, we hypothesised that HBV DNA methylation may result in the unintended and inappropriate methylation of adjacent host CpG islands. Methylation of CpG islands within gene promoter regions could alter the transcriptional activation of genes resulting in biological changes that are responsible for the clinical manifestation of liver disease and cancer development seen in chronic HBV infection.

Genome-wide analysis of host CpG island methylation in patients with chronic HBV infection may identify hypermethylated genes whose abnormal function may help in understanding how chronic HBV infection causes chronic liver inflammation, fibrosis and cancer.

2.2 CLINICAL SETTING OF THE STUDY

This study was conducted at the Liver clinic and research laboratory, Department of Medicine, Groote Schuur Hospital, University of Cape Town (UCT) between February 2010 and August 2013. Groote Schuur Hospital is a tertiary academic institution of UCT. It is a 940 bedded hospital and the clinical service of the Division of Hepatology attends to over 3000 outpatient visits annually with 200 -300 hospital admissions. About 40 – 55 % of these admissions are for procedures such as liver biopsy.

Following informed and signed consent (see **Appendix 28)**, liver tissue samples were collected from patients undergoing liver biopsies as part of their routine clinical management at the hospital. The primary cohort was of patients with chronic HBV

genotypes A and D infection and control cases with autoimmune hepatitis, non-alcoholic fatty liver disease, drug induced liver injury and hepatitis C virus (HCV). The HBV infection was confirmed by serological detection of HBV antigens as well as the detection of HBV DNA in all cases (National Health Laboratory Sciences, Groote Schuur Hospital, UCT, South Africa).

Each sample was split into two parts. One half was put into RNAlater® RNA stabilization reagent (Sigma, SA) for use in DNA, RNA and protein isolation and the other half was put into gluteraldehyde for future electron microscopy work. All the samples were immediately stored at -80°C until isolation. In addition, HCC tissue samples were obtained from formalin-fixed paraffin-embedded (FFPE) liver biopsies from the archives of the National Health Laboratory Sciences, Groote Schuur Hospital, UCT, South Africa. Permission for collection of liver tissue samples for research purposes was obtained from the Health Sciences Research Ethics Committee of the UCT, South Africa (**Ethics Approval Number: 247/2010,** see **Appendix 27)**.

DNA, RNA and protein extraction was done at the Liver Research Laboratories, Groote Schuur Hospital, UCT (see **Appendix 1**). Samples for the thesis microarray work were analysed at the Kusuma School of Biological Science, Indian Institute of Technology, New Delhi (IITD), India. Only FFPE liver tissue derived DNA was used for microarray work. This work was done as part of a postgraduate research exchange visit funded by the UCT Baron Hartley Scholarship, National Research Foundation and The Academy of Sciences for the Developing World fellowship programme for postgraduate research and advanced training. The bisulfite DNA sequencing work was done in the Liver Research Laboratory, Department of Medicine, Groote Schuur Hospital, UCT. The bisulfite DNA sequencing was done using DNA extracted from peripheral blood.

2.3 AIMS OF THE STUDY

The main aim of the study was to investigate for the presence of genome-wide promoter region CpG island methylation profiles in patients with chronic HBV infection and validate these with clinical disease and outcomes.

The specific objectives of the study were to:

1) Investigate for the presence of promoter region CpG island methylation profiles in patients with chronic HBV infection.
2) Correlate the methylated genes identified with known clinical features of liver disease due to chronic HBV infection.
3) Validate the obtained microarray data analysis using bisulfite DNA sequencing on an extended cohort of patients with chronic HBV infection and normal controls.

2.4 STUDIES REPORTED IN THIS THESIS

The overall research plan was divided into two sections that involved investigating for the presence of promoter region CpG islands methylation in patients with chronic HBV infection and validating the findings using bisulfite DNA sequencing (see **Figure 2.1**).

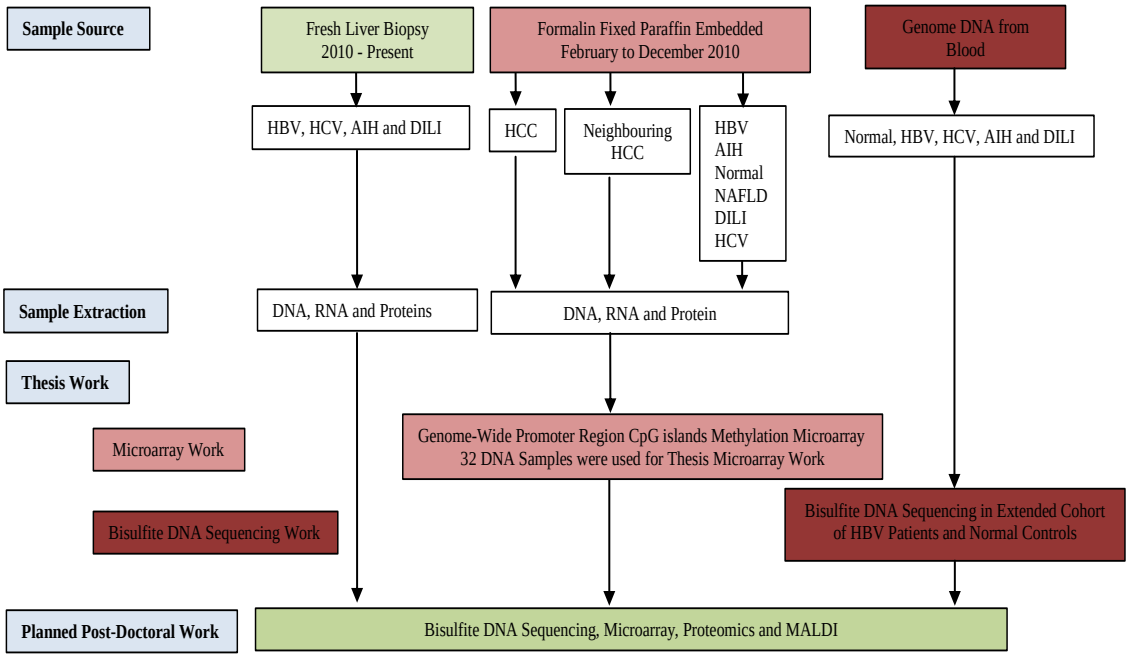

Figure 2.1: The Overall Research Plan Employed in the Study.

The overall research plan was divided into two sections that involved investigating for the presence of promoter methylation across the genome of patients with chronic HBV infection and validation of these findings with bisulfite DNA sequencing work.

Abbreviations: **AIH** - Autoimmune hepatitis, **DILI** - Drug induced liver injury, **DNA** - Deoxyribonucleic acid, **HBV** - Hepatitis B virus, **HCC** - Hepatocellular carcinoma, **HCV** - Hepatitis C virus, **MALDI** - Matrix assisted laser desorption/ionization, **N** - Number.

Chapter 3 describes the genome-wide promoter methylation array work on FFPE tissues from a cohort of patients with chronic HBV infection. FFPE tissues from patients with AIH, HCC and tissues adjacent to resected HCC were used as disease controls. FFPE tissues from patients with no evidence of liver disease were used as normal controls. For microarray analysis, 32 tissue samples were used for the thesis work.

Chapter 4 describes the validation of the promoter microarray data using bisulfite DNA sequencing in the DNA extracted from the blood samples. A validation cohort of HBV patients and normal controls was used for this work in which eleven different genes were investigated. Separately, a highly methylated gene, *Cyclin D3* was used for further validation work in a cohort of chronic HBV infected patients and normal controls.

Chapter 5 discusses and integrates the study findings with the published literature. A hypothesis that integrates the study findings and how they could explain many of the known features of hepatic inflammation, fibrosis, abnormal cell proliferation and cancer seen in chronic liver injury due to HBV infection is presented.

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CHAPTER 3

INVESTIGATION OF GENOME-WIDE PROMOTER REGION CPG ISLAND METHYLATION PROFILES IN PATIENTS WITH CHRONIC HBV INFECTION

3. INTRODUCTION

DNA-methylation analysis assays such as Southern blot, bisulfite DNA sequencing, combined bisulfite restriction analysis (COBRA), methylation specific PCR (MSP), methylation-sensitive single nucleotide primer extension (MS-SNuPE) and electrochemistry have been useful in investigating the presence of epigenetic alterations in human disease. However, they are low-throughput, labour intensive and expensive. The major weakness of these technologies is that they are gene-specific and analyse genes individually at a single-base resolution of the methylome rather than at multiple CpG dinucleotides [1 - 6]. This makes it difficult for researchers to have a full understanding of global epigenetic alterations, which have an important role in influencing human disease progression and cancer development.

Advanced high-throughput microarray-based platforms that are capable of detecting and mapping DNA methylation profiles across the entire genome are continually being developed and improved. Microarray-based platforms are more sensitive and have a high resolution which makes them able to interrogate methylation profiles of multiple genes at a genome-wide scale. They also provide large DNA sequences reads, which makes the data easier to annotate and interpret biologically using various software programmes [7]. Examples of microarray platforms that are available include the Roche Nimblegen®, Affymetrix®, Agilent® and Illumina arrays® [7, 8]. These arrays involve coupling of bisulfite conversion, digestion with methylation-sensitive restriction enzymes and immunoprecipitation of methylcytosine or methyl binding domain proteins procedures with hybridization prior to microarray analysis. The most commonly used standard procedure is differential methylation hybridization [9].

Differential methylation hybridization involves the use of a frequent cutter *MseI* and

a combination of methylation sensitive enzymes such as *BstUI*, *HhaI* and *HpaI*. The digested products are PCR-amplified to generate DNA targets that can be used to investigate methylation profiles on a microarray platform [9 - 11].

The ability of high throughput technologies to analyse methylation profiles at a genome-wide scale was illustrated recently in various methylation analysis studies which discovered the presence of differentially methylated targets in the human genome [13 - 17, 210]. Recently, Zhao et al[12] used the Agilent Custom Human Microarray platform to analyse genome-wide CpG islands promoter methylation profiles in gastric cancer cells. They found novel methylated CpG sites in the gene promoter regions which have not been previously described. Studies using the Illumina Infinium Human Methylation27 BeadChip®, Roche NimbleGen HD2® and Agillent® arrays also found several new patterns of DNA methylation in different diseases and cancers [13 - 17]. These studies illustrate that the microarray platforms can be used as a high-throughput tool to enable more accurate detection of differentially methylated regions across the entire human genome.

GeneChip® Human Promoter 1.0R array is another example of high-throughput technologies and this platform was employed in the work reported in this thesis. It is a high resolution and sensitive tilling array with probes reproducing all known non-repetetive DNA sequences [18, 19]. It interrogates promoter region CpG island methylation profiles, histone proteins interaction and transcription factor binding sites across the entire human genome [218]. It has more than 4.6 million probes per array tilled through over 25,500 human promoter regions. Each promoter region is located within approximately 7.5kb upstream and 2.45kb downstream from the transcription start site (TSS) [218]. The promoter region covered by probes was expanded to include an additional 10kb upstream and 2.5kb downstream of the TSS to include an additional 1300 cancer-associated genes. The probes are 25bp long tilled at a resolution of 35bp intervals with10bp gaps between the oligonucleotides. The promoter regions were annotated by the University of California, Santa Cruz in the National Center for Biotechnology Information (NCBI) human genome assembly (Build 34) using sequence information from Ensembl genes (version21_34d May 14, 2004), RefSeq mRNAs (NCBI GenBank® February 7, 2004) and complete-coding sequence mRNA (NCBI GenBank® December 15, 2003). The arrays contain control

probe sets for both hybridization (e.g *bioA, bioB, bioC, cre* and *dap*) and tilling mRNA transcripts (*dap*, *lys*, *phe*, *thr*, LTP4, LTP6, CAB, RBCL, RCA, RCP1, XCP2, NAC1, PRKASE and TIM) [218].

DNA methylation studies have largely relied on high-quality DNA extracted from unprocessed tissue samples. The difficulty in obtaining unprocessed tissue samples limits the ability to do large studies, particularly of population based-epidemiology. Until recently, the use of formalin fixed paraffin embedded (FFPE) tissues in downstream analyses of methylation and gene expression profiles was not recommended due to heavy degradation and modification of nucleic acids that are cross-linked in amino acid molecules [20, 21]. When using current commercial kits with appropriate protease digestion steps, it is possible to release huge and usable amounts of nucleic acids from FFPE tissues despite degradation [22]. Complete deparaffinisation followed by a series of ethanol washes and proteinase K digestion to reverse formalin fixation and facilitate the release of DNA from the cross-linked protein are some of the techniques used to improve the quality of DNA and RNA recovered from FFPE tissue samples [23].

Human Promoter 1.0R microarray platform allows analysis of DNA extracted from FFPE material, providing many opportunities for large retrospective studies in tissue samples [24, 26]. In this study, some FFPE tissues were successfully used for microarray work. This was attributed to the use of restriction enzymes and PCR amplification which enabled the generation of high quality dsDNA targets for hybridization and microarray.

3.1 STUDY AIMS

The aim of this study was to investigate for the presence of genome-wide promoter region CpG islands methylation in patients with chronic HBV infection.

3.2 MATERIALS

3.2.1 Fresh Liver Biopsy Samples

Sixty-two fresh liver biopsies were collected from patients undergoing liver biopsies as part of their routine clinical management at Groote Schuur Hospital, University of Cape Town (UCT), South Africa. Following informed and signed consent (see **Appendix 28)**, a 1 - 2 mm piece of fresh liver biopsy was cut from obtained liver biopsy specimen and split into two parts. One half was put into RNAlater RNA stabilization reagent for use in DNA, RNA and protein isolation and the other half was put into gluteraldehyde for future electron microscopy work. All the samples were immediately stored at -80°C until isolation.

3.2.2 Formalin-Fixed Paraffin Embedded Liver Biopsy Samples

FFPE liver biopsy samples were used to obtain HCC tissues taken at surgical resection as this was extremely difficult to access from fresh liver biopsies. Two of these samples had biopsies of adjacent liver tissue with no HCC, which were used as controls in the microarray experiment. FFPE liver biopsy samples were also used to obtain liver tissue samples of two cases that were known to have no evidence of liver disease.

One hundred and twelve FFPE tissue samples were retrieved from the archives of the National Health Laboratory Service, Groote Schuur Hospital, UCT, South Africa. These specimens were from patients undergoing routine liver biopsies as part of their clinical mamangement at Groote Schuur Hospital, Cape Town during the period of 2008 - 2010. The study was approved by Health Sciences Research Ethics Committee of UCT, South Africa (**Ethics Approval Number**: **247/2010**, see **Appendix 27**). Based on the samples size calculation, study costs and efficiency, DNA from 32 FFPE samples was used for the microarray work (see also **Chapter 2, Figure 2.1**).

3.3 METHODS FOR SAMPLE PREPARATION AND MICROARRAY ANALYSIS

3.3.1 Nucleic Acids Extraction from Fresh Liver Biopsy Samples

Total genomic DNA, RNA and protein were simultaneously extracted from fresh liver biopsies using TRI-reagent (Sigma Aldrich) according to the manufacturer's protocol (see **Appendix 1**). Briefly, 1mm of liver biopsy tissues was homogenised in TRI-reagent solution. After addition of 1-Bromo-3-chloropropane to the homogenised mixture and centrifugation, the mixture was separated into three phases containing DNA, RNA and protein respectively. The phases were transferred individually into fresh tubes and used to extract DNA, RNA and protein as outlined in **Appendix 1**. The quality of recovered products was quantified using a Nanodrop ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, USA).

3.3.2 Nucleic Acids Extraction from Formalin-Fixed Paraffin Embedded Liver Tissue Samples

In this study, the AllPrep® DNA/RNA FFPE extraction kit (Qiagen, Valencia, CA; Hilden, Germany) was used for simultaneous isolation of both DNA and RNA according to the manufacturer's protocol (see **Appendix 2)**. Briefly four 5 μm-thick sections of FFPE tissue per sample were used. The FFPE sections were deparaffinised in heptane-methanol solutions, rehydrated with ethanol and then centrifuged to obtain pellets for nucleic acids isolation. Xylene can also be used for deparaffinisation; but heptane has been shown to be more convenient and efficient than xylene as it provides a compact sample pellets with good nucleic acids extraction.

Total genomic DNA and RNA was then isolated from the pellets obtained. Samples were lysed, followed by proteinase K digestion and centrifugation at 12000 x g for 10 minutes at 4°C (Eppendorf 5417C Centrifuge, Germany) to obtain a DNA-containing pellet and RNA-containing supernatant. RNA was extracted from the supernatant and kept in storage at -80°C for future use. The DNA-containing pellets were digested again with proteinase K and incubated at 90ºC. After centrifugation, the sample was bound onto a QIAamp MinElute® spin column membrane, washed

and eluted. The eluted DNA sample was stored in aliquots at -80°C. The quality of the DNA samples was determined by agarose gel electrophoresis and quantified using a Nanodrop ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, USA) with absorbance of the DNA read at a wavelength of 260nm (A_{260}) and 280nm (A_{280}).

3.3.3 Generation of Double Stranded DNA Targets

Differential methylation hybridization of DNA was performed based on the technique developed by Huang and Perry for genome-wide DNA methylation analysis in the human genome [27]. The preparation process of DNA targets is illustrated in **Figure 3.1**. The sources of reagents and the protocols employed are outlined in **Appendices 3 - 9**.

Briefly, genomic DNA derived from FFPE tissues was restricted to completion with four-base TTAA cutter, *MseI* restriction enzyme from New England Biolabs, USA (see **Appendices 3** and **4**). The *MseI* enzyme restricts bulk DNA into smaller fragments (<200bp), but leaves larger GC-rich CpG islands fragments relatively intact. The digested DNA is run on 1.5% agarose gel to check the efficiency of digestion. A uniform smear indicates complete digestion. The cleaved ends of the digests were purified using the QIAquick PCR Purification Kit® (Qiagen, USA, **Appendix 3**) according to the manufacturer's instruction as outlined in **Appendix 5**.

The purified cleaved ends of the DNA were ligated to the annealed universal linkers that are meant to enable the subsequent amplification of methylated fragments (Yan et al, 2002). The linkers were prepared by adding H-12 oligonucleotide 5′-TAA TCC CTC GGA- 3′ and H-24 oligonucleotide 5′-AGG CAA CTG TGC TAT CCG AGG GAT-3′ (see **Appendix 6)**. After ligation of the digested DNA to the linkers, PCR amplification was performed to check for successful linker ligation. The PCR product was checked on a 1.5% agarose gel and the smear was visualized using ethidium bromide (10mg/ml). A uniform smear of 100 - 2000 bp in size indicated successful *MseI* digestion and linker ligation. The PCR product was purified using the QIAquick PCR Purification Kit® (Qiagen, USA) as described in **Appendix 5**.

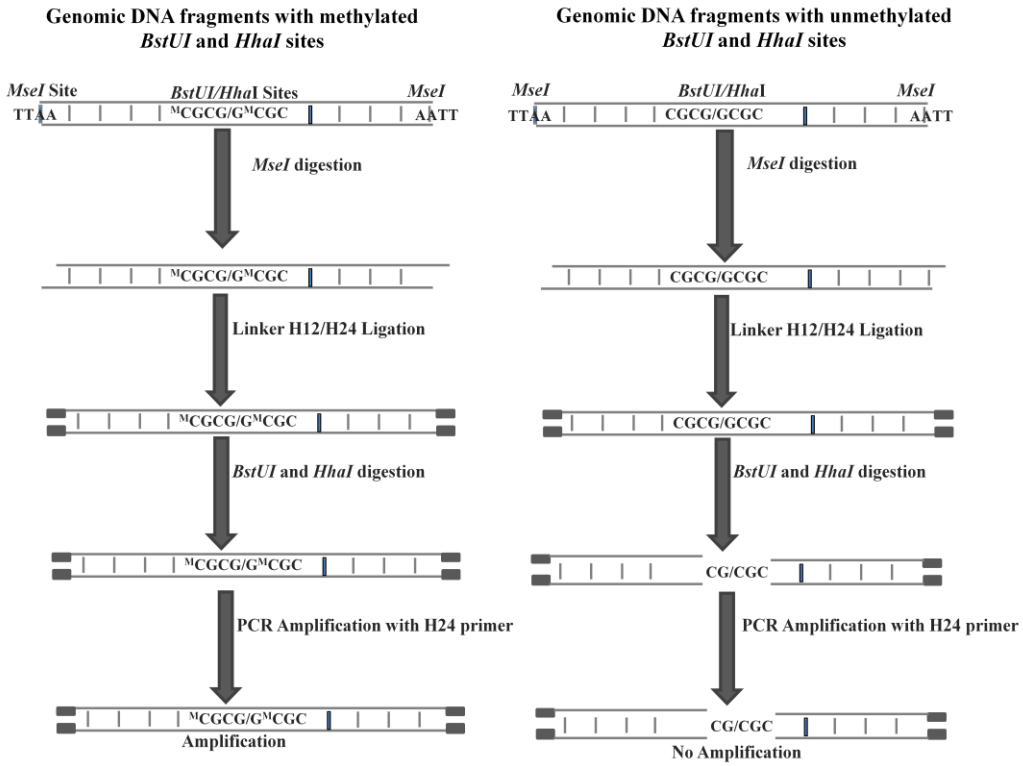

Figure 3.1: Schematic Outline for Generating the DNA Targets.

DNA sample is first digested with a four-base cutter, *MseI* (*TTAA*), an enzyme that restricts bulk DNA into small fragments (<200bp), but preserves larger GC-rich CpG island fragments. These GC-rich fragments are ligated to H12/H24 linkers and sequentially digested with two methylation-sensitive endonucleases, *BstUI* (CGCG) and *HhaI* (GCGC). Only unmethylated DNA fragments are digested by these enzymes, whereas methylated DNA fragments remain intact and are then amplified by PCR using H24-primer.

Abbreviations: C - Cytosine, **G** - Guanine, **T** - Thymine, **A** - Adenine, **DNA** – Deoxyribonucleic acid, **bp** - Base pairs, **PCR** - Polymerase chain reaction, **M** - Methylation.

The purified products were digested with the methylation sensitive endonuclease *BstU1* (New England Biolabs, USA), after which they were digested with the methylation sensitive endonuclease *HhaI* from New England Biolabs, USA. Only genomic DNA fragments containing unmethylated *BstUl* (CGCG) *or HhaI* (GCGC) sites are cut whereas the fragments containing methylated *BstUl* (mCGCG) *or HhaI* (GmCGC) sites remained unaffected from the digestion such that they can be amplified subsequently by PCR [217]. After digestion, the DNA was purified and PCR amplification was carried out. The dUTP was incorporated into the PCR mix for replacement of dTTP during amplification, to enable DNA fragmentation with

Uracil-DNA glycosylase (UDG). The protocols for ligation, PCR amplification and digestion are outlined in **Appendices 7 - 9**.

3.3.4 Fragmentation and Labelling of Double Stranded DNA Targets

After PCR amplification, 7.5 microgram (µg) of the amplified DNA was fragmented and labelled using the Affymetrix® chromatin immunoprecipitation assay protocol [242]. The purpose of fragmentation is to reduce the size of dsDNA fragments to less than 200bp fragments. This facilitates the subsequent step of hybridization onto the microarray chip. The amplified DNA targets were treated with UDG (Affymetrix, Santa Clara California) which breaks down the dsDNA into smaller fragments by removing dUTP residues. Human apurinic/apyrimidinic endonuclease I (APE1) from Affymetrix, Santa Clara, California was used to create a nick in the phosphodiester backbone of the apurinic/apyrimidinic site created by UDG treatment. This enables the labelling of the fragments with biotin. The protocol used for fragmentation and labelling is outlined in **Figure 3.2** and detailed in **Appendix 10** and **11**.

After fragmentation, each fragmented DNA sample was run on 4% agarose gel and bands between 100 - 200 bp were considered proper fragmentation. The fragmented samples were treated with terminal deoxynucleotidyl transferase, recombinant (rTdT) from Affymetrix, Santa Clara, California and labelled with DNA labelling reagent (DLR) also from Affymetrix. This process labelled the 3′ end of the dsDNA fragments with DLR containing the Biotin-11-dXTP Analog that binds to a fluorescent molecule during staining.

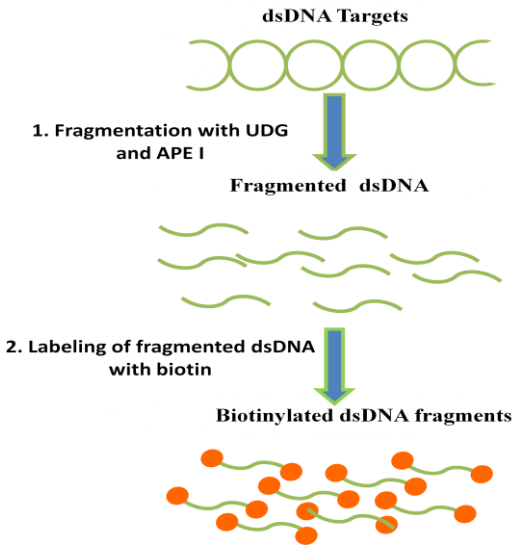

Figure 3.2: Outline of Protocol for Fragmentation and Labelling of DNA Targets.

Amplified DNA was fragmented by treatment with UDG and APE 1 enzymes that remove the dUTP residues and leave the dsDNA in smaller fragments. The resulting DNA fragments were labelled with biotin molecules.

Abbreviations: **dsDNA** - Double stranded deoxyribonucleic acid, **UDG** - Uracil DNA glycosylase, **APE1** - Human apurinic/apyrimidinic endonuclease I.

3.3.5 Hybridization, Washing, Staining and Scanning of Microarray Chips

The biotinylated dsDNA targets were hybridized to the GeneChip® Human Promoter 1.0R array (Affymetrix, Santa Clara, California). This array is a high resolution and sensitivity tilling array with probes reproducing all known non-repetitive DNA sequences. The procedure for hybridization, staining and washing is depicted in **Figure 3.3.** Hybridization was done according to the manufacturer's protocol (see **Appendix 12)**. It was performed for 16 hours at 45°C in the Genechip® Hybridization Oven 640 (Affymetrix, Santa Clara, California) rotating at 60 rpm.

The hybridised probe arrays were stained, washed and scanned. Prior to the staining and washing cycles, the arrays were first registered on the Affymetrix GeneChip Command Console® (AGCC) installed on the computer connected to the microarray platform workstation using AGCC Fluidics Control Software® (Affymetrix, Santa Clara, CA, USA).

The arrays were stained and washed on the Genechip® Fluidics Station 450

(Affymetrix, Santa Clara, CA, USA). The staining reagents had stain 1 containing the fluorescent molecule streptavidin-phycoerythrin (SAPE), which binds to biotin. During a series of staining and washing cycles with anti-SAPE IgG (goat) and biotynylated anti-streptavidin antibody reagents, additional newly deposited biotins bind to SAPE and provides amplified flour that emits light when scanning of arrays.

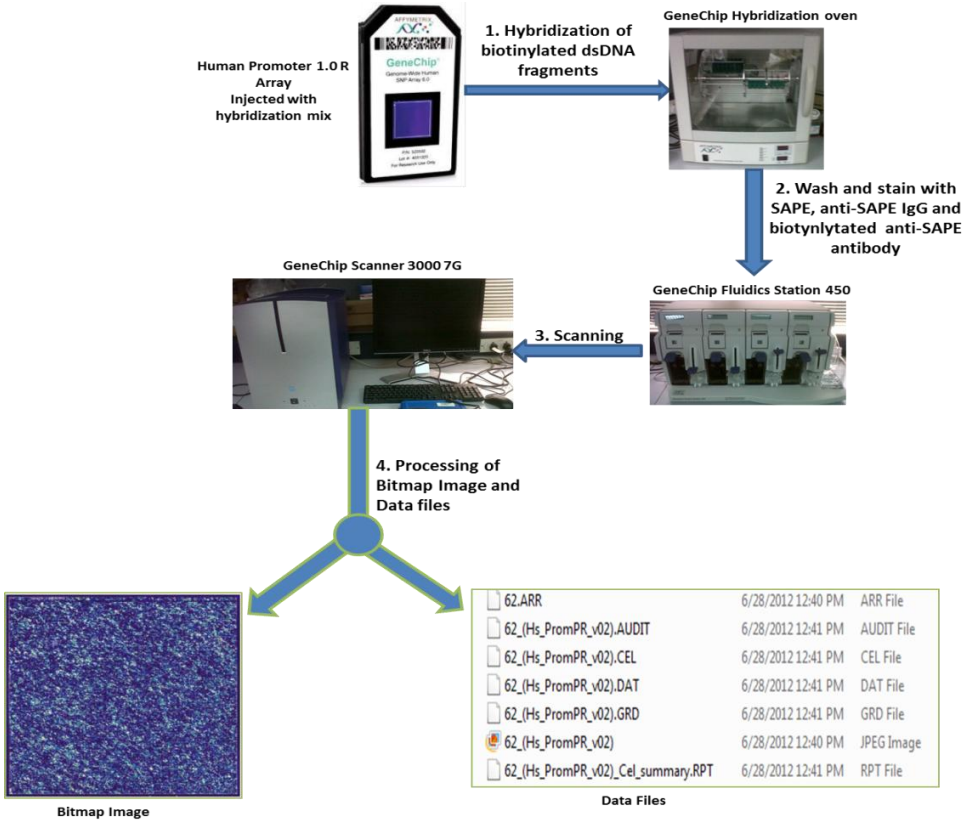

Figure 3.3: Outline of Protocol for Hybridization, Washing, Staining and Scanning.

The chips were hybridized in the Genechip® hybridization oven 640 for 16 hours. The arrays were then stained with reagents containing SAPE molecules with biotin and washed using the Genechip® Fluidics Station 450. The chips were scanned with Genechip® Scanner 3000 7G and the array BitMap images and data files generated were used for data analysis.

Abbreviations: **dsDNA** - Double stranded deoxyribonucleic acid, **SAPE** – Streptavidin phycoerythrin, **IgG** - Immunoglobulin G.

The microarray chips were then scanned with the Genechip® Scanner 3000 7G (Affymetrix, Santa Clara, CA, USA). The scanner contains a confocal laser beam that records the distribution of signals in the arrays. Array BitMap images and raw data files were generated for use in data analysis. The protocols followed for

registering, staining, washing and scanning the probe arrays are described in **Appendices 13 -15.**

3.4 STATISTICAL METHODS AND MICROARRAY DATA ANALYSIS

The algorithm used for data analysis is outlined in **Figure 3.4**. The main stages of analysis were raw data importation into an a microarray data analysis programme, microarray data statistical analysis by sampling groups, determination of methylated sites and biological pathway analysis using gene ontology (GO) terms. The data analysis was performed using the Partek Genomic Suite Software® (PGSS) 6.0 (Partek Incorporated, North America). The PGSS is an advanced microarray data bioinformatics programme that integrates the statistical analysis of microarray data, sampling groups, demographics and GO terms of small or large data sets on an interactive, fast and efficient platform.

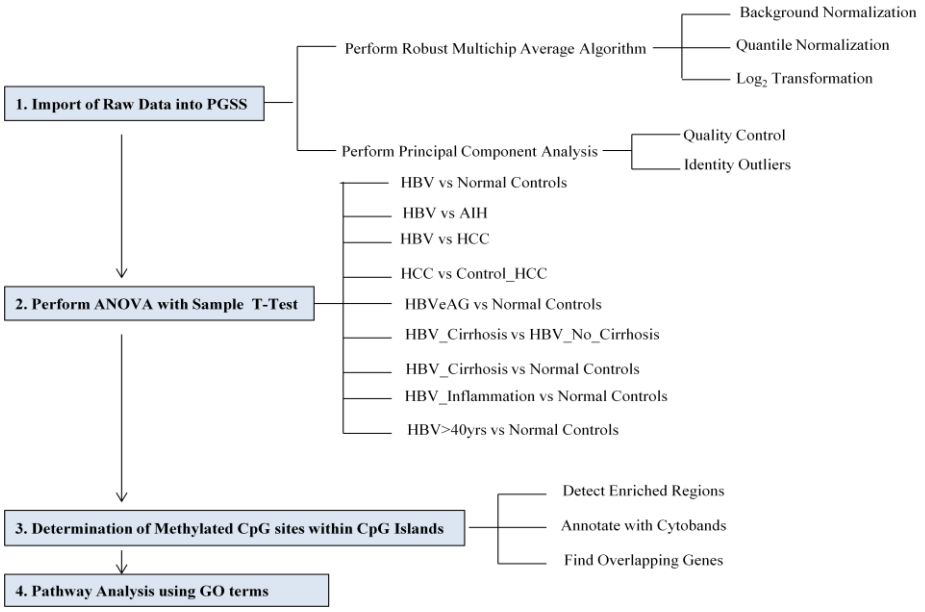

Figure 3.4: Outline of the Workflow used to Analyse the Microarray Data.

Abbreviations: **ANOVA** – Analysis of variance, **AIH** – Autoimmune hepatitis, **CpG** – Cytosine-phosphate-Guanine, **GO** – Gene ontology, **HBV** – Hepatitis B virus, **HBV>40yrs** – Infected HBV patients older than 40 years, **HBV_Cirrhosis** – HBV infection with cirrhosis**, HBV_No_Cirrhosis** – HBV infection without cirrhosis, **HBV_inflammation**- HBV infection with inflammation, **HCC** – Hepatocellular carcinoma, **PGSS** – Partek Genomic Suite Software®.

3.4.1 Data Importation, Normalisation and Background Correction

Raw array data files (CEL files) with methylation measurements generated by GeneChip® Operating Software (GCOS) were imported into PGSS 6.0 and analysed using the PGSS Affymetrix Tilling workflow interface. The human genome GRCh37/hg19 (hg19) assembly was used to analyse the probe data [243]. Hg19 data was used to extract 600bp sequences centred at each probe start position. The imported data were processed using the Robust Multichip Averaging (RMA) algorithm which performs $\log_2$ transformation, background correction and quantile normalization to remove any technical biological batch effects. Principal component analysis (PCA) was done as part of quality control and to identify potential outliers that could significantly affect the data analysis.

The Model-based Analysis of Tilling-arrays (MAT) algorithm in PGSS 6.0 was used to analyse the probe signals for significant differences that could have biological effects [18]. The MAT algorithm performs a robust trimmed mean of *t*-values that removes outliers and noisy effects across all normalised probe intensities in a window of 600bp in length. It scores regions for chromatin immunoprecipitation enrichment which allows for the calculation of robust p-value cut-off values and false discovery rate calculations. Positive MAT scores would represent hypermethylation in for example, HBV tissues relative to another group being compared to them whereas negative MAT scores would represent hypomethylation relative to the comparison group.

3.4.2 Statistical Analysis by Disease Categories

To compare enriched regions in different groups, an Analysis of Variance (ANOVA) for the different diagnoses of HBV infection, AIH, HCC and normal controls was created by applying the t-test and fold change calculated at $\log_2$-scale. The ANOVA spreadsheet generated contained p-values, fold change and T-statistic values. A p-value of ≤ 0.05 was considered significant. A positive T-statistic value when comparing HBV cases to another group, meaning that HBV cases have a higher average intensity than the comparison group. When it is negative, HBV cases have a lower average intensity than the comparison cases. The ANOVA results were used to

identify the enriched regions that are differentially methylated between different groups.

The MAT algorithm was used to asses methylation quantified by the MAT score using the default or corrected probe on a 600bp window with at least 10 probes. The analysis generated a spreadsheet of an enriched regions list with MAT scores appearing as either positive or negative. Positive MAT scores when comparing HBV cases to normal controls mean that the identified region in HBV cases is hypermethylated compared to normal controls.

3.4.3 Detection of Methylated Regions

To detect differentially methylated regions, the enriched regions were annotated to their corresponding overlapping regions using the PGSS gene annotation tool against hg19 transcripts (Affymetrix U133_Plus_2_na24.hg19.-transcript.csv file [246]). In addition, methylated genes and cytobands (chromosomal locations) located 7kb upstream and 2.45kb downstream from transcription start site (TSS) were also annotated. For purposes of this thesis, the analysis of significantly differentially methylated genes was focused on the region 100bp upstream from TSS, with high MAT scores and cut-off p-values of ≤ 0.05 (see **Figure 3.5)**.

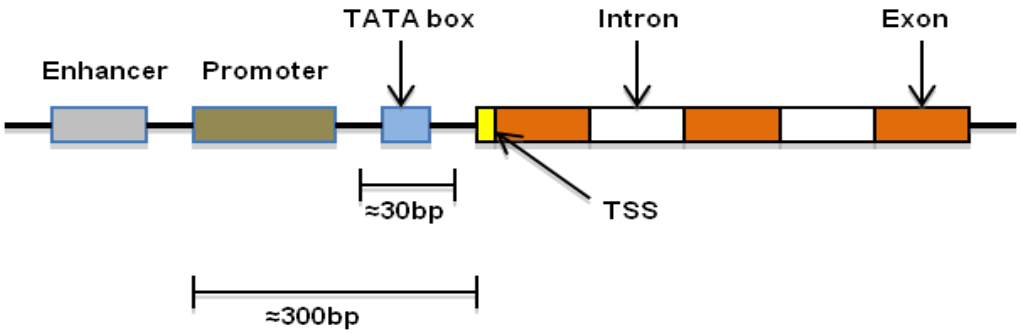

Figure 3.5: Typical Structure of Eukaryotic Gene Illustrating Region Analysed.

The core promoter is located upstream of a gene and has a TATA sequence that is required to properly initiate gene transcription. In this thesis work only data on methylated CpG island regions in the promoter region starting within 100bp of the transcription start site were analysed. If methylated, this region would affect gene transcription the most.

Abbreviations: **bp** – base pairs, **TSS** – Transcription start site.

Methylation of promoter CpG island regions of various cellular genes when comparing HBV to AIH, HCC or normal controls was analysed. The distance between a region of interest to the TSS and the overlap percentage of the region with nearest genes were reported. The length of overlap divided by the length of the transcript was also calculated and this represented the percentage of overlap with a region or gene (see **Figure 3.6)**. The percentage overlap with a gene of the methylated CpG islands that were analysed were all zero (see **Figure 3.6A**), meaning that the methylated region did not overlap with a gene and was entirely within the promoter region upstream to TSS.

Only methylated CpG islands located as illustrated in **Figure 3.6A, B** and starting within 100bp of TSS were analysed for this thesis presentation. For example, the methylated sequence that was used for the *CCND3* gene was located and started 100bp **upstream** from the TSS as illustrated in **Figure 3.6B** and was 573 base pairs in size. Another sequence that was 13121bp **downstream** from the TSS as illustrated in **Figure 3.6D** and 676bp in size was not used as this was located within the gene region.

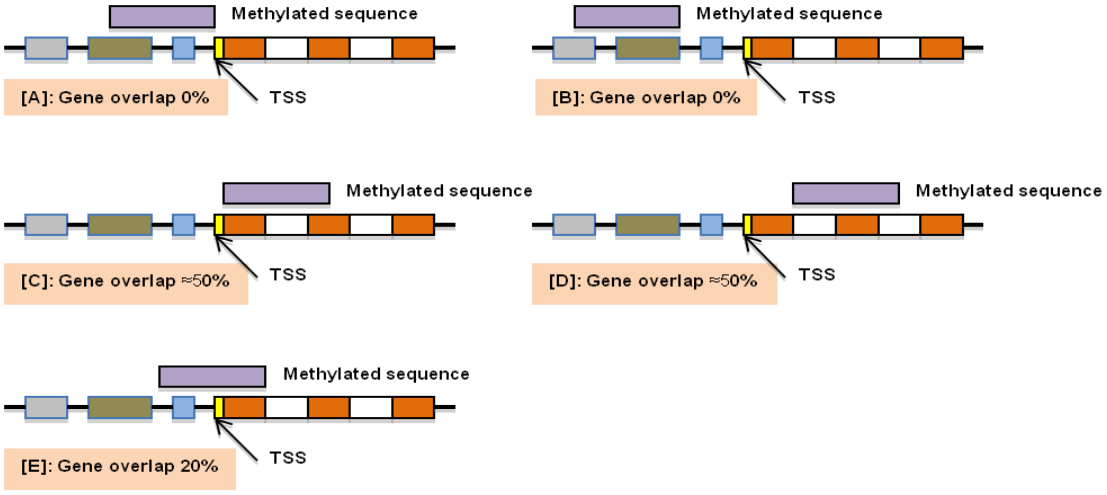

Figure 3.6: Illustration of the Gene Overlap Annotation.

A and B: Percentage overlap with a gene is 0%, meaning that the methylated region does not overlap with a gene and is entirely within the promoter region upstream of TSS. **C and D**: The methylated region overlap with the nearest gene is 50% and is located entirely downstream of TSS. **E:** The methylated region overlap is 20% with the nearest gene while the rest is located upstream of TSS (within promoter region).

Abbreviations: **TSS** – Transcription start site.

3.4.4 Pathway Analysis

Pathway analysis using GO terms was used to determine the biological significance of the identified genes. Genes were tested using Fisher's exact test and analysis was restricted to pathways with more than 2 genes. The p-value of less than 0.01 was used to filter methylated gene promoter CpG island regions to increase the significance. Significant pathways with high enrichment scores and significant p values less than 0.01 were reported.

3.4.5 Cohort Analysis

The histology of the samples was scored using the histological activity index of Ishak et al[220]. Samples were categorised as cirrhotic if the fibrosis score was ≥5/6 and assessed as having significant hepatic inflammation if the inflammation grade score was ≥7/18.

The demographic and clinical patient variables were analysed using STATA 13 (College Station, Texas, USA). Categorical binary data were summarised as counts and percentages while categorical ordinal data was summarised as medians and inter-quartile values. Ordinal numerical data was summarised as medians and inter-quartile values and continuous numerical data as means and standard deviations.

The investigation of possible associations between categorical variables was performed using the Pearson Chi-squared test and Fishers' exact test where appropriate. A two-sided p value of <0.05 was considered significant.

3.5 RESULTS

3.5.1 Sample Patient Cohort Profile

The characteristics of the microarray patient cohort are described in **Table 3.1**. 56.25% of the cohort was of mixed ancestry race, 62.5% were male and 40.62% were cirrhotic. The mean age at sampling was 36.8 years. Most of the samples were from HBV infected patients and 18.75% were HCC tissues.

Table 3.1: Characteristics of Microarray Patient Cohort

Cohort Characterisation	**Number = 32**
	Mean (SD)
Age (years)	36.8 (15.4)
	Median (IQR)
ALT (0 – 40 u/L**)**	115.5 (30.5-202.5)
	Number (%)
Male	20 (62.5)
Race	
Caucasian	4 (12.5)
Mixed race	18 (56.25)
Indian	3 (9.38)
Black African	7 (21.88)
Diagnosis	
Normal	2 (6.25)
Hepatitis B virus	18 (56.25)
Autoimmune Hepatitis	4 (12.5)
Hepatocellular Carcinoma	6 (18.75)
Corresponding HCC Control	2 (6.25)
Histology	
Liver Cirrhosis (HAI Score ≥ 5/6)	13 (40.62)

Abbreviations: % - Percentage, **ALT** - Alanine aminotransferase, **HAI** - Histology Activity Index**, HCC** - Hepatocellular carcinoma, **IQR** - Inter-quantile range, **u/L –** International unit per litre **SD** – Standard deviation.

3.5.2 Generation of dsDNA Targets, DNA Fragmentation and Labelling

Analysis with 1.5% agarose gel was used to confirm the presence of successful digestion, DNA linker-ligation, PCR amplification of methylated sites and generation of dsDNA fragments of less than 200bp for use in microarray analysis (see **Figure 3.7**). The successful digestion of genomic DNA with *MseI* restriction enzyme is shown in **Figure 3.7A.** A uniform smear pattern of between 50 – 2000 bp indicates successful and complete digestion.

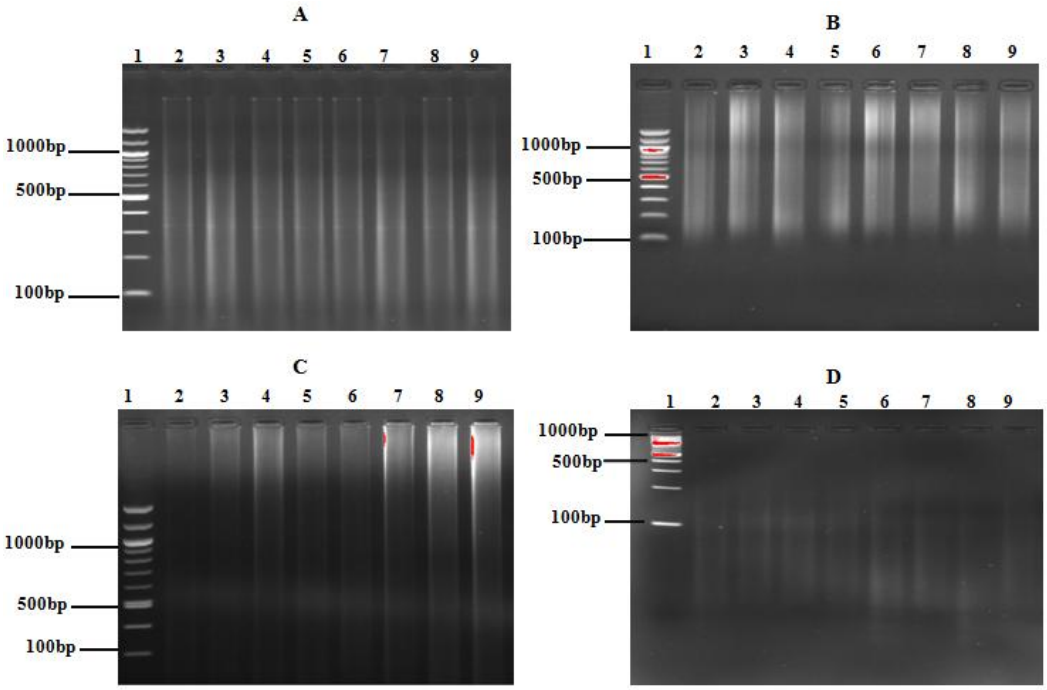

Figure 3.7: Agarose Gel Confirmation of Successful dsDNA Targets Generation and Fragmentation.

DNA aliquots were run on 1.5% agarose gel. **A**: DNA after *MseI* digestion, **B**: DNA after ligation with linkers and PCR amplification, **C**: DNA after digestion with methylation sensitive endonucleases *BstUI* and *HhaI* and PCR amplification, **D**: dsDNA targets after fragmentation for confirmation of successful fragmentation.

Lane 1: Molecular weight marker, **Lane 2**: Sample ID_62, **Lane 3**: Sample ID_66, **Lane 4**: Sample ID_68, Lane **5**: Sample ID_69, Lane **6**: Sample ID_71, **Lane 7**: Sample ID_72, **Lane 8**: Sample ID_73 and **Lane 9**: Sample ID_74.

Abbreviations: **dsDNA** - Double-stranded deoxyribonucleotides, **PCR** - Polymerase chain reaction.

The agarose gel in **Figure 3.7B** shows successful DNA-linker ligation. This is confirmed by the presence of a smear pattern between 100 - 2000 bp. **Figure 3.7C** shows successful PCR amplification of fragments with methylated *BstUI* and *HhaI* sites. Successful fragmentation of dsDNA into smaller fragments by the removal of dNTP residues is shown in **Figure 3.7D** gel with the expected DNA fragments of smear patter being less than 200bp in size.

3.5.3 Principal Component Analysis of Microarray Data

Principal component analysis (PCA) after background correction for the differences in probe signal intensities and DNA methylation profiles showed clustering of the samples by diagnosis (see **Figure 3.8B**). There was one significant outlier of a HBV sample.

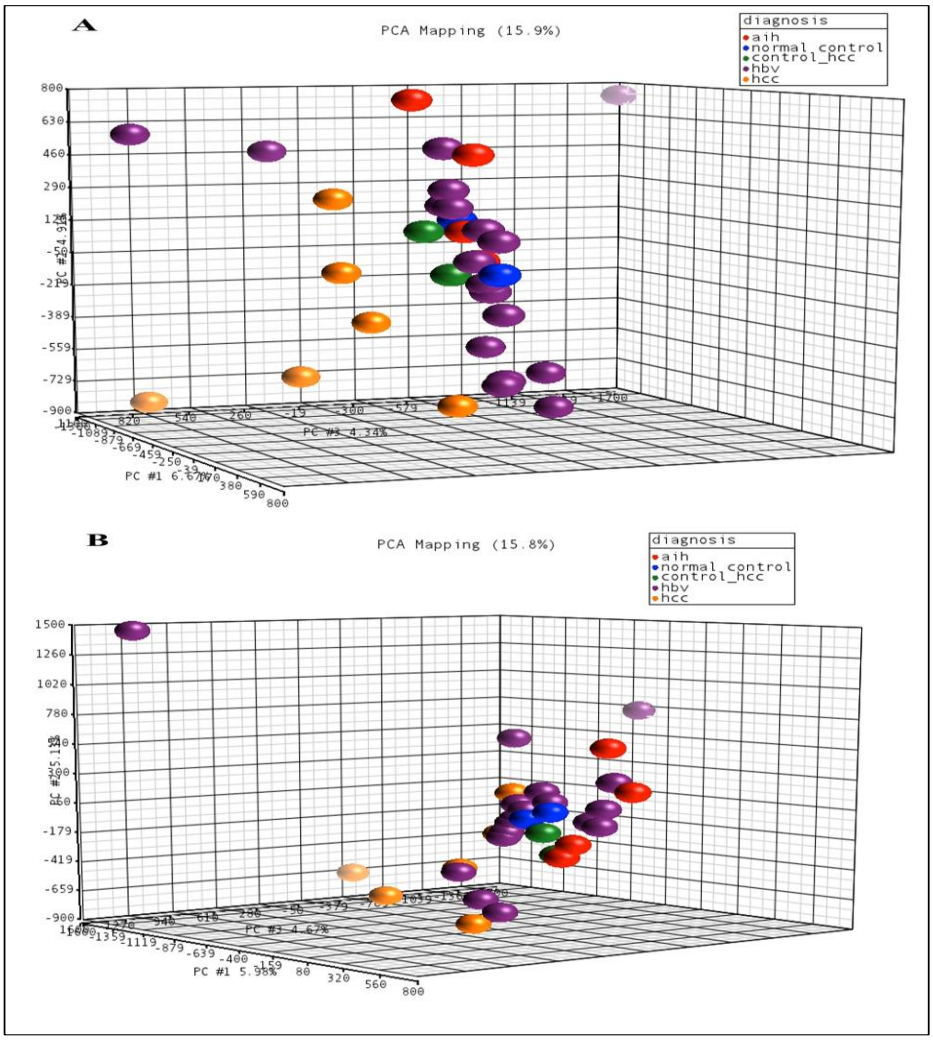

Figure 3.8: Principal Component Analysis of Microarray Data Samples.

The data for each sample type is represented by different colours in circles. HBV tissue samples are presented in purple, AIH in red, HCC in orange, corresponding HCC controls in green and normal controls in blue. **A** represents PCA analysis before background correction and **B** represents PCA analysis after background correction.

Abbreviations: **AIH** - Autoimmune hepatitis, **HBV** - Hepatitis B virus, **HCC** - Hepatocellular carcinoma, **PCA** - Principal Component Analysis.

The data for each sample type is represented by different colours in circles. HBV tissue samples are presented in purple, AIH in red, HCC in orange, corresponding HCC controls in green and normal controls in blue. **Figure 3.8A** shows clustering of HCC tissue samples from HBV, AIH, Control-HCC and Normal tissue samples which are closely clustered and have less variation across individual samples. After background correction, PCA shows more close clustering of tissue samples across various groups with one HBV samples still straying away from the HBV samples (see **Figure 3.8B**).

3.5.4 Profile of Promoter Region CpG Islands Methylation Within 100bp of the TSS in the Cohort

The methylation profile of promoter regions within 100bp upstream of the transcription start site in the cohort used for microarray is shown in **Figure 3.9**. There were no hypermethylated core promoter regions within 100bp upstream of the TSS in chromosomes X and Y while chromosomes 11 and 19 had the highest number of hypermethylated genes at 6 genes each.

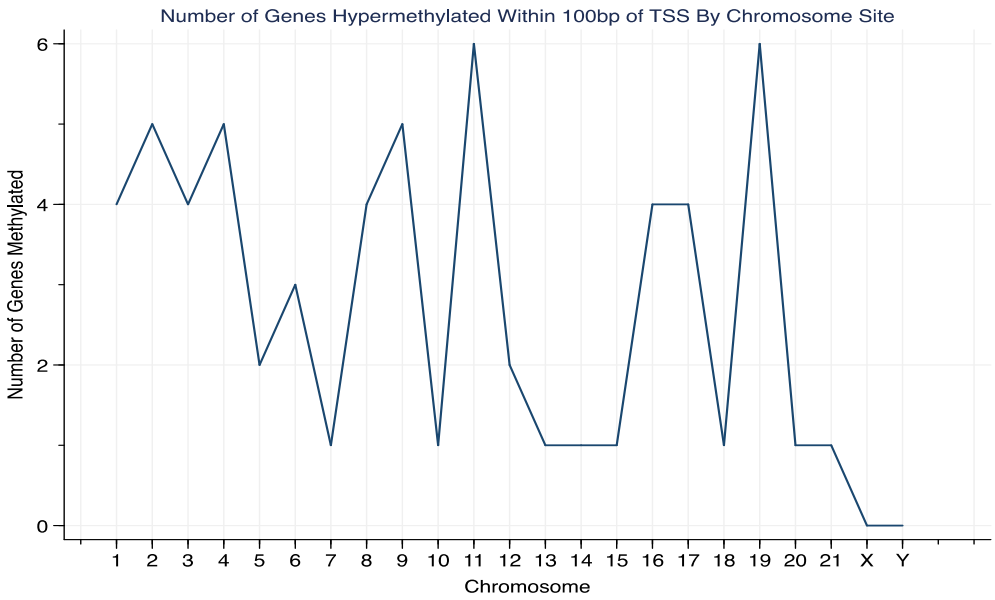

Figure 3.9: Profile by Chromosome of Hypermethylated Gene Promoter Regions within 100bp of TSS in the Microarray Cohort

Abbreviations: **bp** – Base pair, **TSS** – Transcription Start Site.

The chromosomes with promoter region hypermethylation in the different comparison groups of chronic HBV infected patients are shown in **Figure 3.10** and compared to the profile of the whole microarray cohort shown in **Figure 3.10.A**. When all the patients with chronic HBV infection were compared to normal controls, there was hypermethylation across the genome with most of it occurring in chromosome 9 (see **Figure 3.10.B**). In HBV infected patients older than 40 years, there was a slight increase in gene hypermethylation in chromosome 19 when compared to normal controls (see **Figure 3.10.C**).

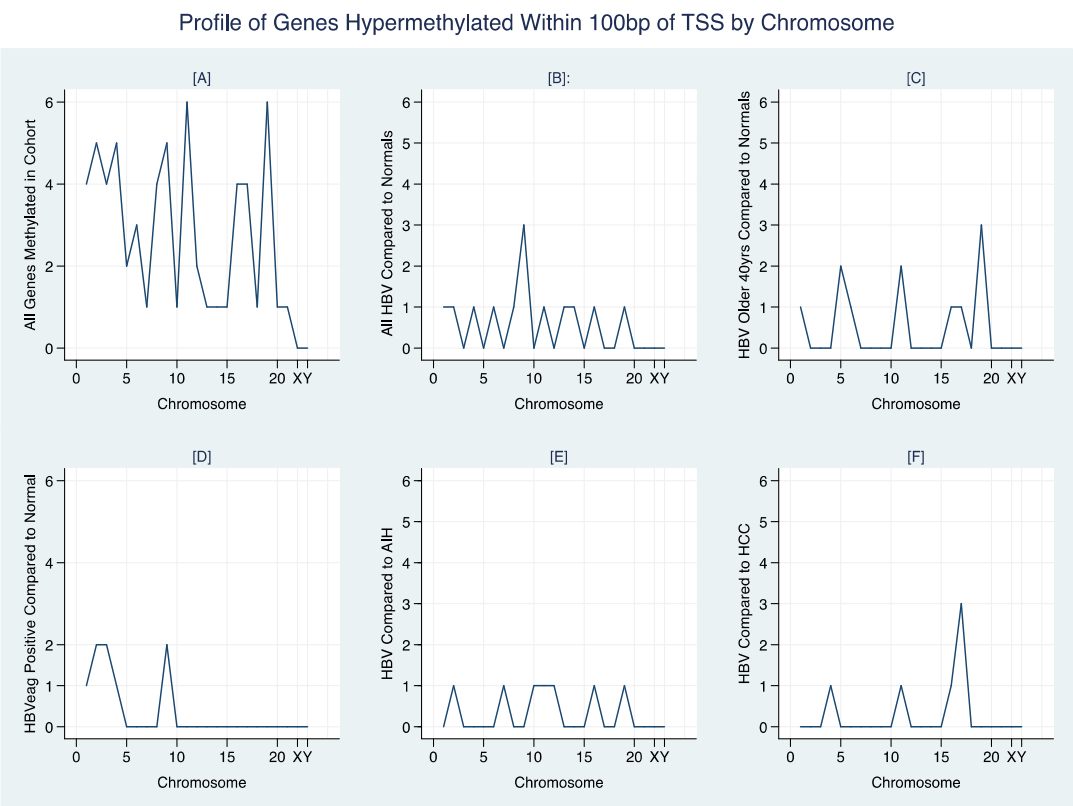

Figure 3.10: Profile by Chromosome of Hypermethylated Gene Promoter Regions within 100bp of TSS in the HBV Infected Patients Compared to Controls

Abbreviations: **AIH** - Autoimmune hepatitis, **bp** - Base pair, **HBV** - Hepatitis B virus, **HBVeag** - Hepatitis B Virus e antigen, **HCC** - Hepatocellular Carcinoma, **TSS** - Cytosine Transcription Start Site

In HBV e antigen positive patients compared to normal controls, most of the gene hypermethylation was in chromosomes 2, 3 and 9 (see **Table 3.8D**), HBV patients compared to autoimmune hepatitis patients had hypermethylation in a varied number

of chromosomes (see **Table 3.8.E**) but when compared to hepatocellular carcinoma cases, most of the gene hypermethylation was in chromosome 17 (see **Table 3.8.F**).

3.5.5 Methylation Profiles of Promoter CpG Islands in HBV Infection

The age at diagnosis was not significantly different between the diagnostic groups (see **Table 3.2**). There were significantly more male patients with HBV infection compared to HCC patients (**p = 0.02)**. There were no male patients with AIH. The prevalence of cirrhosis was similar in all the groups and only 27.8% of the HBV patients were HBV e antigen positive. AIH has significantly higher ALT levels (**p = 0.03**) compared to HCC patients. In the HBV infected cohort, 55% were genotype A and 45% genotype D. There were no cases of genotype B, C or E documented.

Table 3.2: Cohort Characteristics by Diagnosis

	HBV	**HCC**	**AIH**	**P value**
	Median (IQR)	**Median (IQR)**	**Median (IQR)**	
Age (years)	34.2 (13.1)	43.5 (19.9)	37.5 (9.9)	N/S
ALT (0 – 40 u/L)	103 (30 - 317)	51.5 (26 - 124)	331.5 (212 – 377.5)	0.03^{K}
αFP (0 – 7 u/L)	3 (2.2 – 4)	2.8 (1 – 3.2)	2.6 (1.7 – 3.45)	N/S
Log_{10} viral DNA	3.75 (2.3 – 6)	0	N/A	N/A
	Number (%)	**Number (%)**	**Number (%)**	
Male	13 (72.2)	4 (66.7)	0	0.02^{P}
Liver cirrhosis (HAI ≥ 5/6)	8 (44.4)	2 (33.3)	2 (50)	N/S
All HBV cases	18 (100)	1 (16.7)	N/A	N/A
HBeAg positive	5 (27.8)	0	N/A	N/A

Abbreviation: **αFP** - Alpha fetoprotein, **AIH** - Autoimmune hepatitis, **DNA** - Deoxyribonucleic acid, **HAI** – Histology activity index, **HBeAg** – Hepatitis B virus e antigen, **HBV** - Hepatitis B virus, **HCC** – Hepatocellular carcinoma, **IQR** - Inter-quantile range, **N** – Number, **N/A** - Not applicable, **N/S** – Not significant, **$P = 0.03^{k}$** - AIH compared to HCC, **$P = 0.02^{P}$** - AIH compared to HBV

The results of promoter CpG island methylated sites less than 100bp upstream of the TSS are summarised in **Tables 3.3 - 3.11**. A positive MAT score in a group represents hypermethylation relative to the comparison group, while negative MAT score denotes relative hypomethylation.

In all the HBV infected patients, 13 genes were hypermethylated within 100bp upstream of the TSS in the core promoter region while 17 hypomethylated relative to normal controls (see **Table 3.3).** The most hypermethylated promoter CpG islands were *olfactory receptor family 8 subfamily B member 1* (*OR8B4*), *GA binding protein transcription factor beta subunit 2* (*GABPB2*), *interleukin 11 receptor alpha*

(IL11RA), par-6 partitioning defective 6 homolog alpha (*PARD6A*) and c*yclin D3* (*CCND3*) genes.

Table 3.3: Methylation Profile of Gene Promoter CpG Islands in HBV Patients Compared to Normal Controls

Distance to TSS	MAT-score	Gene Title	Gene Name	Cytoband	Transcript ID	P-value
Hypermethylated in HBV relative to Normal (Hypomethylated in Normal)						
-9	2.6209	OR8B4	Olfactory receptor, family 8, subfamily B, member 1	11q24.2	NM_001005196	0.00136
-21	2.2629	LNX2	Ligand of numb-protein X 2	13q12.2	NM_153371	0.00737
-26	2.2016	NAIF1	Nuclear apoptosis inducing factor 1	9q34.11	NM_197956	0.00959
-30	2.2772	DEFT1P	Defensin theta 1 pseudogene	8p23.1	NR_036686.1	0.00684
-57	2.3022	GABPB2	GA binding protein transcription factor beta subunit 2	1q21.3	NM_144618	0.00615
-60	2.2185	PTGR2	Prostaglandin reductase 2	14q24.3	NM_152444	0.00899
-60	2.1953	TUBA4A	Tubulin alpha 4a	2q35	NM_024463	0.00986
-65	2.2753	MEPE	6kDa matrix extracellular phosphoglycoprotein	4q22.1	NM_001184694	0.00689
-71	2.2795	NCRNA00085	Non-protein coding RNA 85	19q13.41	NM_024463	0.00679
-84	2.2505	EXOSC3	Exosome component 3	9p13.2	NM_016042	0.00777
-86	2.4618	IL11RA	Interleukin 11 receptor, alpha	9p13.3	NM_001142784	0.00291
-91	2.3311	PARD6A	Par-6 partitioning defective 6 homolog alpha	16q22.1	NM_016948	0.00532
-100	2.2651	CCND3	Cyclin D3	6p21.1	NM_001136126	0.00730
Hypomethylated in HBV relative to Normal (Hypermethylated in Normal)						
-7	-2.5454	PAX1	Paired box1	20p11.22	NM_006192	0.0056
-10	-3.3904	LYPD5	LY6/PLAUR domain containing 3	19q13.31	NM_001031749	0.00045
-22	-4.0944	NBPF1	Neuroblastoma breakpoint family member 1	1p36.13	NM_017940	<0.0001
-23	-2.3635	MIR644	MicroRNA 644	20q11.22	NR_030374	0.00881
-27	-2.3305	ABHD1	Abhydrolase domain containing 1	2p23.3	NM_032604	0.00961
-31	-2.5429	MIR138-2	MicroRNA 138-2	16q13	NR_029680	0.00573
-32	-5.0814	LOC100189589	DICTN1antisense RNA 1	2p13.1	NM_032604	<0.0001
-45	-3.1406	PLAC8	Placenta specific 8	4q21.22	NM_032604	0.00104
-59	-2.3770	PRSS53	Protease serine 53	16p11.2	NR_029680	0.00859
-63	-2.4615	OR52I1	Olfactory receptor, family 52, subfamily I, member 1	11p15.4	NR_024463	0.00711
-63	-2.3890	NSMAF	Neutral sphingomyelinase activation associated factor	8q12.1	NM_001130716	0.00836
-65	-2.3328	TMEM80	Transmembrane protein 80	11p15.5	NM_001039503	0.00954
-73	-2.4788	NAT6	N-acetyltransferase 6	3p21.31	NM_001200018	0.00687
-73	-3.0574	GSN	Gelsolin	9q33.22	NM_00114477	0.00133
-77	-3.4419	CARHSP1	Calcium regulated heat stable protein 1	16p13.2	NM_174940	0.00039
-83	-2.6742	UBB	Ubiquitin B	17p11.2	NM_018955	0.00402
-100	-2.4788	HYAL3	Hyaluronoglucosaminidase 3	3p21.31	NM_001200029	0.00687

Abbreviation: **HBV -** Hepatitis B virus, **MAT** – Model based analysis of tilling array, **TSS -** Transcription start site

When compared to AIH, only 7 gene promoter CpG islands were hypermethylated within 100bp upstream of TSS in HBV infected patients while 9 genes were hypomethylated (see **Table 3.4**). Some of these were pseudogenes including *RNA polymerase transcription factor homolog pseudogene 3* (*RRN3P3*). Amongst 3 gene promoter CpG islands hypermethylated in this category were *nuclear apoptosis inducing factor 1* (*NAIF1*), *exosome component 3* (*EXOSC3*) and *interleukin 11 receptor, alpha (IL11RA)* which are located in chromosome 9 (see **Table 3.4)**. Chromosome 9 was shown to be highly methylated in HBV infected patients as compared to normal controls (see **Figure 3.10B**).

Table 3.4: Methylation Profile of Gene Promoter CpG Islands in HBV Compared to AIH Patients

Distance to TSS	MAT-score	Gene Title	Gene Name	Cytoband	Transcript ID	P-value
Hypermethylated in HBV relative to AIH (Hypomethylated in AIH)						
-23	3.04796	NUDT13	Nucleoside diphosphate linked moiety X type motif 13	10q22.1	NM_015901	0.0030
-29	3.14933	KRT77	Keratin 77	12q13.13	NM_175078	0.0021
-31	3.12476	RRN3P3	RNA polymerase transcription factor homolog pseudogene 3	16p12.2	NM_027460	0.0023
-41	3.22968	PTOV1	Prostate tumour overexpressed gene 1	19q13.33	NM_017432	0.0014
-51	2.85175	TRIM77	Tripartite motif-containing 77	11q14.3	NM_001146162	0.0057
-54	3.00349	TRIP12	Thyroid hormone receptor interactor 12	2q36.3	NM_004238	0.0034
-67	2.99707	TRPV5	Transient receptor potential cation channel subfamily v member 5	7q34	NM_019841	0.0035
Hypomethylated in HBV relative to AIH (Hypermethylated in AIH)						
-3	-3.12636	OR8B8	Olfactory receptor family 8 subfamily member 1	11q24.2	NM_012378	0.0039
-4	-4.96107	IFIT1B	Interferon induced protein with tetratricopeptide repeats 1B	10q23.31	NM_001010987	<0.0001
-38	-2.84555	PRAME	Preferentially expressed antigen in melanoma	22q11.22	NM_206955	0.0081
-16	-3.30101	PRAMEF20	PRAME family member 20	1p36.21	NM_001099852	0.0024
-27	-2.96886	LOC729080	Glycine cleavage system protein H (aminimethyl carrier) pseudogene	5q31.3	NM_033244	0.0059
-38	-2.83998	C6orf97	Chromosome 6 open reading frame 97	6q25.1	NM_025059	0.0082
-38	-3.11647	DPEP3	Dipeptidase 3	16q22.1	NM_022357	0.0039
-51	-4.07695	CXorf57	Chromosome X open reading frame 57	Xq22.3	NM_018015	0.0003
-58	-3.02184	TUBGCP5	Tubulin, gamma complex associated protein 5	15q11.2	NM_052903	0.0051

Abbreviation: **AIH** - Autoimmune hepatitis, **HBV** - Hepatitis B virus, **MAT** - Model-based analysis of tilling array, **TSS** - Transcription start site

Compared to HCC cases, there were 5 genes hypermethylated in HBV patients, some of which were pseudogenes. These include *coiled-coil domain containing 144B (CCDC144B)* and *keratin 42* (*KRT42P*) pseudogenes. Nine genes were hypomethylated in HBV relative to HCC patients (see **Table 3.5**).

Table 3.5: Methylation Profile of Gene Promoter CpG Islands in HBV Compared to HCC Patients

Distance to TSS	MAT-score	Gene Title	Gene Name	Cytoband	Transcript ID	P-value
Hypermethylated in HBV relative to HCC (Hypomethylated in HCC)						
-11	3.02086	FAM106C	Family with sequence similarity 106, member C	17p11.2	NR_026810	0.0066
-17	4.31789	PRSS41	Protease serine 41	16p13.3	NM_001135086	0.0002
-35	3.2606	CCDC144B	Coiled-coil domain containing 144B (pseudogene)	17p11.2	NR_036647	0.0033
-43	3.29559	KRT42P	Keratin 42 pseudogene	17q21.2	NM_033415	0.0031
-44	2.97	MS4A13	Membrane-spanning 4-domains, subfamily A, member 13	11q12.2	NM_001012417	0.0076
Hypomethylated in HBV relative to HCC (Hypermethylated in HCC)						
-1	-3.78691	HSPA1A	Heat shock 70kDa protein 1A	6p21.33	NM_005345	0.0012
-2	-3.21856	DYRK4	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 4	12p13.32	NM_003845	0.0049
-10	-3.07763	DGCR11	Di-George syndrome critical region gene 11	22q11.21	NR_024157	0.0070
-17	-3.32986	PAOX	Polyamine oxidase (exo-N4-amino)	10q26.3	NM_207127	0.0037
-34	-3.11781	MIR1293	MicroRNA 293	12q13.12	NR_031625	0.0063
-44	-3.01747	PATE3	Prostate and testis expressed 3	11q24.2	NM_001129883	0.0082
-51	--3.11473	ZMYND12	Zinc finger, MYND-type containing 12	1p34.2	NM_032257	0.0064
-51	-2.94263	HTR3B	5-Hydroxytryptamine (serotonin) receptor 3B, ionotropic	11q23.2	NM_006028	0.0099
-56	-3.02223	FAM173A	Family with sequence similarity 173, member A	16P13.3	NM_023933	0.0081

Abbreviation: **HBV** - Hepatitis B virus, **HCC** - Hepatocellular carcinoma, **MAT** - Model-based analysis of tilling array, **TSS** - Transcription start site

When HCC tissues were compared to their neighbouring liver tissues, hypermethylation was present in the promoter CpG islands of 6 genes within 100bp upstream of TSS (see **Table 3.6**). The most hypermethylated were *retintis pigmentosa 1* (*RP1*), *prolyl 4-hydroxylase transmembrane* (*P4HTM*) and *prostate and testis expressed 3* (*PATE3*). The neighbouring tissues had 6 genes hypermethylated.

Table 3.6: Methylation Profile of Gene Promoter CpG Islands in HCC Tissues Compared to Corresponding Tissues with no HCC

Distance to TSS	MAT-score	Gene Title	Gene Name	Cytoband	Transcript ID	P-value
Hypermethylated in HCC relative to Control HCC (Hypomethylated in Control HCC)						
-21	3.00403	RP1	Retinitis pigmentosa 1 (autosomal dominant)	8q12.1	NM_006269	0.0033
-26	2.75559	ALAS1	Aminolevulinate, delta-, synthase 1	3p21.2	NM_000688	0.0078
-30	2.75367	KRBA2	KRAB-A domain containing 2	17p13.1	NM_213597	0.0078
-33	3.23248	P4HTM	Prolyl 4-hydroxylase, transmembrane (endoplasmic reticulum)	3p21.31	NM_177938	0.0014
-44	3.18627	PATE3	Prostate and testis expressed 3	11q24.2	NM_001129883	0.0017
-51	2.68891	SCARB2	Scavenger receptor class B, member 2	4q21.1	NM_001204255	0.0098
Hypomethylated in HCC relative to Control HCC (Hypermethylated in Control HCC)						
-13	-2.6544	FBXO39	F-box protein 39	17p13.1	NM_153230	0.0086
-15	-3.05614	SNX11	Sorting nexin 11	17q21.32	NM_013323	0.0022
-29	-3.43835	OR11H2	Olfactory receptor, family 11, subfamily H, member 2	14q11.2	NM_001197287	0.0011
-41	-3.04642	DNAJC14	DNAJ (Hsp40) homolog, subfamily C, member 14	12q13.2	NM_032364	0.0031
-47	-3.05614	FAM106C	Family with sequence similarity 106, member C, pseudogene	17p11.2	NM_026810	0.0029
-69	-2.63247	LOC151534	Ladybird homeobox 2	2p13.1	NM_024606	0.0090

Abbreviation: **HBV** - Hepatitis B virus, **HCC** – Hepatocellular carcinoma, **MAT** - Model-based analysis of tilling array, **TSS** - Transcription start site

HBV e antigen positive patients cases had 10 genes with promoter CpG islands hypermethylation compared to normal controls (see **Table 3.7).** The most methylated were *Fibroblast growth factor receptor-like 1* (*FGFRL1*), *NACC family member 2, BEN and BOP* (*POZ*) *domain containing* (*NACC2*), *Non-protein coding RNA 85* (*NCRNA00085*), *Deoxyribosnucleic II beta* (*DNASE2B*), *Polycomb group ring finger 1* (*PCGF1*), *Nuclear apoptosis inducing factor 1* (*NAIF1*) and *Tubulin alpha 4 A* (*TUBA4A*).

Table 3.7: Methylation Profile of Gene Promoter CpG Islands in Patients who are HBV e Antigen Positive Compared to Normal Controls

Distance to TSS	MAT-score	Gene Title	Gene Name	Cytoband	Transcript ID	P-value
Hypermethylated in HBeAg relative to Normal (Hypomethylated in Normal)						
-15	2.11849	SNORA7B	Small necleolar RNA, H/ACA box 7B	3q21.3	NR_002992	0.0093
-25	2.20809	NACC2	NACC family member 2, BEN and BTP (POZ) domain containing	9q34.3	NM_144653	0.0066
-35	2.19158	DNASE2B	Deoxyribonuclease II beta	1p31.1	NM_058248	0.0071
-42	2.15965	PCGF1	Polycomb group ring finger 1	2p13.1	NM_032673	0.0079
-53	2.1286	PA2G4	Proliferation associated 2G4	12q13.2	NM_006191	0.0089
-55	2.30551	FGFRL1	Fibroblast growth factor receptor-like 1	4p16.3	NM_001004358	0.0044
-71	2.18293	NCRNA00085	Non-protein coding RNA 85	19q13.41	NR_024330	0.0074
-84	2.13735	NAIF1	Nuclear apoptosis inducing factor 1	9q34.11	NM_197956	0.0086
-96	2.15182	TUBA4A	Tubulin alpha 4A	2q35	NM_006000	0.0082
-102	2.11849	SNORA7B	Small nucleolar RNA, H/ACA box 7B	3q21.3	NR_002992	0.0093
Hypomethylated in HBeAg relative to Normal (Hypermethylated in Normal)						
-7	-3.01632	PAX1	Paired-box 1	20p11.22	NM_006192	0.0008
-8	-2.23403	OR6C6	Olfactory receptor family 6, subfamily C, member 6	12q13.2	NM_001005493	0.0093
-10	-3.27096	LYPD5	LY6/PLAUR domain containing 5	19q13.31	NM_001031749	0.0003
-21	-2.28038	MIR573	MicroRNA 573	20q11.22	NR_030299	0.0082
-22	-3.20013	NBPF1	Neuroblastoma breakpoint family member 1	1p36.13	NM_017940	0.0004
-23	-2.31912	MIR644	MicroRNA 644	20q11.22	NR_030374	0.0074
-34	-2.2307	ATP5S	ATP synthase H+ transporting mitochondrial Fo complex subunit	14q21.3	NM_001003803	0.0094
-37	-2.28793	SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein	5p15.33	NM_004168	0.0079
-41	-3.65721	CARHSP1	Calcium regulated heat stable protein 1	16p13.2	NM_014316	<0.0001
-52	-3.52326	POU5F1P3	POU class 5 homeobox 1 pseudogene 3	12p13.31	NR_036440	<0.0001
-59	-2.54622	PRSS53	Protease serine 53	16p11.2	NM_001039503	0.0037
-60	-2.21473	NTSR1	Neurotensin receptor type 1	20q13.33	NM_002531	0.0098
-63	-2.28793	NSMAF	Neutral sphingomyelinase activation associated factor	8q12.1	NM_001144772	0.0081
-64	-2.23439	PODNL1	Podocan-like 1	19p13.12	NM_001146255	0.0093
-68	-2.21249	DEFB109P1	Defensin beta 109 pseudogene 1	8p23.1	NR_024044	0.0099
-72	-2.39771	MTUS1	Microtubule associated tumour suppressor 1	8p22	NM_020749	0.0059
-77	-2.24916	GRID2IP	Glutamate receptor ionotropic delta 2 interacting protein	7P22.1	NM_001145118	0.0089
-81	-2.48487	PCDHA9	Protocadherin alpha -9	5q35.3	NM_014005	0.0044
-81	-2.37638	SLC34A1	Solute carrier family 34 (sodium phosphate) member 1	5q35.3	NM_003052	0.0062
-85	-2.7105	XKR8	XK, Kell blood group complex subunit-related family member 8	1p35.3	NM_018053	0.0022
-92	-2.46624	CGB8	Chorionic gonadotropin beta polypeptide 8	19q13.33	NM_033183	0.0047
-94	-2.27505	IKZF3	IKAROS family zinc finger 3	17q12	NM_183228	0.0083
-97	-2.25635	CYB5D1	Cytochrome b5 domain containing 1	17p13.1	NM_144607	0.0087

Abbreviation: **HBV** - Hepatitis B virus, **HBeAg** – Hepatitis B e antigen, **MAT** - Model-based analysis of tilling array, **TSS** - Transcription start site

Cirrhotic HBV infected patients had 6 genes hypermethylated compared to those without cirrhosis (see **Table 3.8**). The most methylated were *Small nuclear RNA C/D box 48* (*SNORD48*), *Small Cajal body-specific RNA 10* (*SCARNA10*) and *Transmembrane protein 41A* (*TMEM41A*). The non-cirrhotic HBV cases had 19 genes with promoter CpG islands hypermethylation.

Table 3.8: Methylation Profile of Gene Promoter CpG Islands in Cirrhotic HBV Compared to Non-Cirrhotic HBV Patients

Distance to TSS	MAT-score	Gene Title	Gene Name	Cytoband	Transcript ID	P-value
Hypermethylated in HBV with Cirrhosis relative to HBV without Cirrhosis (Hypomethylated in HBV without Cirrhosis						
-1	2.87222	NPM2	Nucleophosmin/Nucleoplasmin 2	8p21.3	NM_182795	0.0029
-15	3.14211	SNORD48	Small nucleolar RNA C/D box 48	6p21.33	NR_002745	0.0013
-21	3.18015	SCARNA10	Small Cajal body-specific RNA 10	12p13.31	NR_004387	0.0011
-35	2.75282	SAMHD1	Sterile alpha motif and HD domain containing protein 1	20q11.23	NM_015474	0.0046
-40	2.70982	LAMA1	Laminin alpha 1	18p11.23	NM_005559	0.0052
-67	3.06345	TMEM41A	Transmembrane protein 41A	3q27.2	NM_080652	0.0016
Hypomethylated in HBV with Cirrhosis relative to HBV without Cirrhosis (Hypermethylated in HBV without Cirrhosis						
-16	-3.09943	PRAMEF20	Preferentially expressed antigen in melanoma	1p36.21	NM_001099852	0.0012
-17	-3.19839	KIAA1524	KIAA1542	3q13.13	NM_020890	0.0008
-26	-2.45359	TMEM9	Transmembrane protein 9	1q32.1	NM_016456	0.0008
-27	-4.02128	CBLC	Cbl proto-oncogene E3 ubiquitin protein ligase C	19q13.32	NM_001130852	<0.0001
-30	-2.55635	C6orf174	Chromosome 6 open reading frame 74	6q22.33	NM_001005177	0.0089
-33	-2.4094	MYH9	Myosin 9	22q12.3	NM_002473	0.0098
-35	-2.60874	OR52R1	Olfactory receptor, family 52, subfamily I, member 1	11p15.4	NM_001005177	0.0054
-45	-2.73847	ELAVL2	Embryonic lethal abnormal vision Drosophila-like 2	9p21.3	NM_001171195	0.0035
-57	-3.24346	LNX2	Ligand of numb-protein X 2	13q12.2	NM_153371	0.0007
-64	4.04884	OST4	Oligosaccharyltransferase 4 homolog	2p23.3	NM_001134693	<0.0001
-65	-2.82446	MEPE	Matrix extracellular phosphoglycoprotein	4q22.1	NM_001184694	0.0026
-67	-3.47922	NDUFC1	NADH dehydrogenase ubiquinone 1, subcomplex unknown 1	4q31.1	NM_002494	0.0002
-83	-2.6658	GUCY2C	Guanylate cyclise 2C	12p12.3	NM_004963	0.0004
-85	-3.005	HPX	Hermopexin	11p15.4	NM_000613	0.0015
-87	-2.65857	CCDC72	Coiled-coil domain containing protein 72	3p21.31	NM_015933	0.0046
-95	-2.54305	MICALL2	MICAL-like protein 2	7p22.3	NM_182924	0.0065
-101	-3.0063	S1PR5	Sphingosine-1-phosphate receptor 5	19p13.2	NM_001166215	0.0015
-104	-2.43833	C9orf46	Chromosome 6 open reading frame 46	9p24.1	NM_001012279	0.0062
-105	-4.09363	KCNA3	Potassium voltage gated channel, shaker-related subfamily member 3	1p13.3	NM_002232	<0.0001

Abbreviation: **HBV** – Hepatitis B virus**, MAT** - Model-based analysis of tilling array, **TSS** -Transcription start site

When comparing cirrhotic HBV patients with normal controls, cirrhotic HBV cases had 8 genes with promoter CpG islands hypermethylation (see **Table 3.9**). In normal controls, there were 16 genes with promoter CpG islands hypermethylation.

Table 3.9: Methylation Profile of Gene Promoter CpG Islands in Cirrhotic HBV Patients Compared to Normal Controls

Distance to TSS	MAT-score	Gene Title	Gene Name	Cytoband	Transcript ID	P-value
Hypermethylated in HBV with Cirrhosis relative to Normal (Hypomethylated in Normal)						
-12	2.27614	THRAP3	Thyroid hormone receptor associated protein 3	1p34.3	NM_005119	0.0079
-29	2.44828	LOC285441	Uncharacterised	4q35.2	NM_033900	0.0037
-28	2.34464	UBXN1	UBX domain protein 1	11q12.3	NM_015853	0.0061
-33	2.3729	KCNJ15	Potassium inwardly- rectifying channel subfamily J, member 15	21q22.13	NM_170737	0.0053
-36	2.40318	LRRC28	Leucine rich repeat containing 28	15q26.3	NM_144598	0.0046
-55	2.39852	DEFT1P	Defensin theta 1 pseudogene	8p23.1	NR_036686.1	0.0047
-60	2.25535	TUBA4A	Tubulin alpha 4a	2q35	NM_006000	0.0088
-84	2.24223	NAIF1	Nuclear apoptosis inducing factor 1	9q34.11	NM_197956	0.0094
Hypomethylated in HBV with Cirrhosis relative to Normal (Hypermethylated in Normal)						
-6	-3.33412	OR5AU1	Olfactory receptor, family 5, subfamily AU, member 1	14q11.2	NM_001004731	0.0072
-6	-3.33412	LENG1	Leukocyte receptor cluster member 1	19q13.42	NM_024316	0.0004
-7	-3.04332	PAX1	Paired box 1	20p11.22	NM_006192	0.0012
-9	-3.17101	PLAC8	Placenta 8	4q21.22	NM_00113071	0.0008
-10	-3.09667	LYPD5	LY6/PLAUR domain containing 5	19q13.31	NM_00103174	0.0010
-21	-2.38239	MIR573	MicroRNA-573	4p15.2	NR_030299	0.0076
-27	-2.47331	CBLC	Cbl proto-oncogene E3 ubiquitin protein ligase C	19q13.32	NM_012116	0.0061
-29	-2.73699	TMEM80	Transmembrane protein 80	11p15.5	NM_174940	0.0029
-31	-2.34636	NSMAF	Neutral sphingomyelinase activation associated factor	8q12.1	NM_00114477	0.0084
-32	-5.06774	LOC100189589	DICTN1antisense RNA 1	2p13.1	NR_024463	<0.0001
-63	-2.31751	LOC650368	Asparagine-linked glycosylation 1-like pseudogene	11p15.4	NR_024248	0.0090
-76	-2.39296	TMEM25	Transmembrane protein 25	11q23.3	NM_001144034	0.0074
-86	-3.26291	IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	8p11.21	NM_001556	0.0005
-87	-3.07784	MIR4267	MicroRNA-4267	2q13	NR_036225	0.0011
-89	-3.22882	SCARB2	Scavenger receptor class B, member 2	4q21.1	NM_001204255	0.0006
-103	-3.91033	CCDC56	Coiled-coil domain containing 56 (pseudogene)	17q21.31	NM_001040431	<0.0001

Abbreviation: **HBV** - Hepatitis B virus, **MAT** - Model-based analysis of tilling array, **TSS** - Transcription start site

HBV infected patients with hepatic inflammation had 5 genes hypermethylated within 100bp of the TSS in the core promoter region (see **Table 3.10)**. The most methylated were *NACC family member 2, BEN and BOP* (*POZ*) *domain containing* (*NACC2*), *uncharacterised gene* (*LOC285441*), *MicroRNA-527* (*MIR527*), *defensin theta 1 pseudogene* (*DEFT1P*) and *nuclear apoptosis inducing factor 1* (*NAIF1*). In normal cases, there were 18 genes with hypermethylation of promoter region CpG islands.

Table 3.10: Methylation Profile of Gene Promoter CpG Islands in HBV Patients with Inflammation Compared to Normal Controls

Distance to TSS	MAT-score	Gene Title	Gene Name	Cytoband	Transcript ID	P-value
Hypermethylated in HBV with Inflammation relative to Normal (Hypomethylated in Normal)						
-25	2.7026	NACC2	NACC family member 2, BEN and BTP (POZ) domain containing	9q34.3	NM_144653	0.0026
-28	2.45859	MIR527	MicroRNA-527	19q13.42	NR_030219	0.0064
-64	2.36857	LOC285441	Uncharacterised	4q35.2	NR_033900	0.0090
-84	2.363	NAIF1	Nuclear apoptosis inducing factor 1	9q34.11	NM_197956	0.0093
-92	2.45859	DEFT1P	Defensin theta 1 pseudogene	8p23.1	NR_036686.1	0.0066
Hypomethylated in HBV with Inflammation relative to Normal (Hypermethylated in Normal)						
-7	-2.33096	PAX1	Paired box 1	20p11.22	NM_006192	0.0081
-10	-3.12202	LYPD5	LY6/PLAUR domain containing 5	19q13.31	NM_00103174	0.0006
-29	-2.32991	GTPBP5	GTP binding protein 5 (putative)	20q13.33	NM_015666	0.0081
-35	-3.564	BCAR3	Breast cancer anti-oestrogen resistance 3	1p22.1	NM_003567	0.0001
-40	-3.14107	NAT6	N-acetyltransferase gene	3p21.31	NM_001200018	0.0006
-42	-3.14107	HPGD	Hydroxyprostaglandin dehydrogenase 15 – (NAD)	4q34.1	NM_000860	0.0065
-44	-3.09335	POLA2	Polymerase (DNA directed) alpha 2	11q13.1	NM_002689	0.0007
-55	-3.06265	CGB8	Chorionic gonadotropin, beta polypeptide 8	19q13.33	NM_033183	0.0007
-63	-2.34125	NSMAF	Neutral sphingomyelinase activation associated factor	8q12.1	NM_001144772	0.0078
-67	-3.14107	HYAL3	Hyaluronoglucosaminidase 3	3p21.31	NM_001200029	0.0006
-73	-3.03573	GSN	Gelsolin	9q33.2	NM_001127665	0.0008
-80	-2.34706	CBX8	Chromobox homolog 8	17q25.3	NM_020649	0.0077
-83	-2.35959	UBB	UBX domain protein 1	17p11.2	NM_018955	0.0073
-85	-3.01734	KCNK2	Potassium inwardly- rectifying channel subfamily k, member 2	1q41	NM_001017424	0.0009
-89	-3.03112	SCARB2	Scavenger receptor class B, member 2	4q21.1	NM_005506	0.0008
-96	-2.4994	RECQL	RecQ protein-like DNA helicase Q1-like	12p12.1	NM_032941	0.0048
-97	-2.26522	PCDHA1	Protocadherin alpha 1	5q31.3	NM_031411	0.0098
-103	-3.67078	CCDC56	Coiled-coil domain containing 56	17q21.31	NM_001040431	<0.0001

Abbreviation: **HBV** - Hepatitis B virus, **MAT** - Model-based analysis of tilling array, **TSS** - Transcription start site

HBV infected patients older than 40 years were compared to normal controls (see **Table 3.11**). Older HBV infected patients had 11 genes hypermethylated compared to normal controls. The most methylated includes *coiled-coil domain containing 127* (*CCDC127*), *TBC1 domain family member 17* (*TBC1D17*), *MicroRNA 527* (*MIR527*), *regulator of G protein signalling 14* (*RGS14*) and *small nucleolar RNA, H/ACA box 52* (*SNORA52*). There were 19 genes hypermethylated in Normal relative to HBV infected patients older than 40 years.

Table 3.11: Methylation Profile of Gene Promoter CpG Islands in HBV Patients Older than 40 years Compared to Normal Controls

Distance to TSS	MAT-score	Gene Title	Gene Name	Cytoband	Transcript ID	P-value
Hypermethylated in HBV Patients > 40 years relative to Normal (Hypomethylated in Normal)						
-1	2.51712	CCDC127	Coiled-coil domain containing 127	5p15.33	NM_145265	0.0054
-9	2.39954	OR8B4	Olfactory receptor family 8, subfamily B, member 4	11q24.2	NM_001005196	0.0832
-17	2.3663	THEM95	Transmembrane protein 95	17p13.1	NM_198154	0.0098
-20	2.41936	TBC1D17	TBC1 domain family, member 17	19q13.41	NM_024682	0.0079
-28	2.64899	MIR527	MicroRNA 527	19q13.42	NR_030219	0.0032
-30	2.47682	FANCE	Fanconi anaemia complementation group E	6p21.31	NM_021922	0.0032
-30	2.61364	RGS14	Regulator of G protein signalling 14	5q35.3	NM_006480	0.0064
-48	2.57335	PPP1R8	Protein phosphatise 1 regulatory subunit 8	1p35.3	NM_014110	0.0044
-71	2.40713	NCRNA00085	Non-coding RNA protein	19q13.41	NR_024330	0.0083
-91	2.39208	PARD6A	Par-6 partitioning defective 6 homolog alpha (*C.elegans*)	16q22.1	NM_016948	0.0088
-92	3.11802	SNORA52	Small nucleolar RNA, H/ACA box 52	11p15.5	NR_002585	0.0004
Hypomethylated in HBV Patients > 40 years relative to Normal (Hypermethylated in Normal)						
-6						
-7	-3.15795	LENG1	Leukocyte receptor cluster member 1	19q13.42	NM_024316	0.0010
-10	-2.41437	PAX1	Paired box 1	20p11.22	NM_006192	0.0085
-17	-3.35125	LYPD5	LY6/PlAUR domain containing 5	19q13.31	NM_001031749	0.0006
-27	-3.00631	MIR4267	MicroRNA 4267	2q13	NR_036225	0.0016
-27	-2.54886	CBLC	Cbl proto-oncogene E3 ubiquitin ligase C	19q13.32	NM_012116	0.0058
-31	-2.60717	ABHD1	Abhydrolase domain containing 1	2p23.3	NM_032604	0.0049
-32	-2.49835	MIR138-2	MicroRNA 138-2	16q13	NR_029680	0.0068
-34	-2.47766	SLC30A8	Solute carrier, family 30 (zinc transporter), member 8	8q24.11	NM_001172811	0.0072
-41	-3.02306	GSN	Gelsolin	9q33.2	NM_198252	0.0015
-46	-2.46938	LOC401010	Nucleolar complex associated 2 homolog	2q21.1	NR_002826	0.0073
-49	-3.15419	UBB	Ubiquitin B	17p11.2	NM_018955	0.0010
-55	-2.50097	AP2B1	Adaptor-related protein complex 2, beta 1 subunit	17q12	NM_001282	0.0067
-59	-3.2507	CGB8	Chorionic gonadotropin beta polypeptide 8	19q13.33	NM_033183	0.0007
-65	-2.56456	HTN3	Histatin 3	4q13.3	NM_000200	0.0056
-67	-2.79753	TMEM80	Transmembrane protein 80	11p15.5	NM_174940	0.0030
-96	-5.33245	LOC100189589	DICTN1antisense RNA 1	2p13.1	NR_024463	<0.0001
-98	-2.51545	RECQL	RecQ protein-like (DNA helicase Q1-like)	12p12.1	NM_032941	0.0064
-103	-2.47126	NSMAF	Neutral sphingomyelinase activation associated factor	8q12.1	NM_001144772	0.0073
	-5.33245	CCDC56	Coiled-coil domain containing protein	17q21.31	NM_001040431	0.0002

Abbreviation: **>** - Older than, **HBV** – Hepatitis B virus, **MAT** - Model-based analysis of tilling array, **TSS** - Transcription start site

Genes that were shown to have significant promoter region CpG island hypermethylation within 100bp upstream of TSS in HBV infected patients were summarised in **Table 3.12**. The major functional groups of these genes are related to cyclin dependent kinase, growth factors, regulation of hepatic metabolism, tumour suppressors, transcription regulators, splicing of mRNA, vitamin D receptor (VDR) binding, ubiquitination and pseudogenes. Most of these hypermethylated genes are involved in various signalling pathways including those of HBV infection with HCC calcium, TGF Beta, tuberculosis, cell adhesion, tight junction, mRNA surveillance, MAP kinase, cell cycle, viral carcinogenesis, PPAR gamma, type 2 diabetes mellitus, apoptosis and HTLV-1 infection (see **Appendices 26.A – O**).

Table 3.12: Summary of Genes with Promoter Region CpG Island Hypermethylation and their Clinical Significance in HBV Infection

Genes	Physiological Functions	Related Diseases
A. Cyclin-Dependent Kinase		
CCND3	Liver regeneration; Wound healing process	Liver fibrosis; HCC suppression
PARD6A	Protein kinase C binding; Asymmetrical cell division	Defects in *Caenorhabditis elegans*
B. Growth Factors		
FGFRL1	Negatively regulate cell growth; Tissue regeneration; Healing	Liver fibrosis; HCC suppression
PARD6A	Transforming growth factor β receptor signaling pathway	Defects in *Caenorhabditis elegans*
C. Metabolism Regulators		
PTGR2	Prostaglandin metabolism; Immune function	Autoimmune disease; Type 2 diabetes mellitus
TRPV5	Calcium and phosphorus metabolism	Rickets; Osteoporosis; Hyperparathyroidism
D. Tumor Suppressors		
GABPB2	Cell adhesion; Immune function	Unknown
NACC2	Negatively regulates apoptosis	Unknown
NAIF1	Induction of cell apoptosis	Gastric cancer
PA2G4	Cell proliferation; Cell cycle arrest	Cancer
PARD6A	Cell cycle arrest, proliferation and apoptosis	Metastatic carcinoma
PATE3	Induces cell cycle arrest and apoptosis	Glioma cells
PCGF1	Cell cycle progression and proliferation	B-cell lymphoma
PTOV1	Enhances cell Proliferation	Prostate and bladder malignancies
PTGR2	Cell proliferation and apoptosis	Unknown
TBC1D17	Inhibits cell transformation and proliferation	Unknown
TUBA4A	Regulates cell adhesion	Unknown
E. Transcription Regulators		
EXOSC3	Ribosomal transcription	Unknown
GABPB2	Initiates transcription via RNA polymerase 1	Gastric cancer; inflammation
RRNP3	Initiates transcription via RNA polymerase II	Unknown
PA2G4	Negative regulation of transcription	Cancer
PCGF1	DNA transcription regulator	B-cell lymphoma
PTOV1	DNA Replication and transcription regulator	Prostate and bladder cancers
F. Regulation of RNA Splicing		
EXOSC3	RNA and mRNA metabolic process	Unknown
SNORD48	snoRNA processing and binding	Cancer
THRAP3	Regulates mRNA processing	Hyperthyroidism; Hypercalcemia
G. Genes Encoding Ubiquitin Proteins		
LNX2	E3 ubiquitin ligase activity	Breast cancer
PA2G4	Histone H2A monoubiquitination	Unknown
TRIP12	Histone H2A ubiquitination	Unknown
UBXN1	E3 ubiquitin ligase activity	Unknown
H. Vitamin D -Related Genes		
THRAP3	Vitamin D receptor binding	Hyperthyroidism; Hypercalcemia
TRPV5	Transport vitamin D-induced calcium ion	Hyperparathyroidism
I. Pseudogenes		
RRNP3	Ribosomal transcription	Colorectal cancer
J. Genes that interact with HIV Proteins		
THRAP3	Interact with HIV-1 Protease and Gag Proteins	HIV-1
TUBA4A	Interact with HIV-1 Tat and Rev Proteins	HIV-1

3.6 DISCUSSION

Using the GeneChip® Human Promoter 1.0R array (Affymetrix, Santa Clara, California), this study has investigated the presence of genome wide promoter CpG island methylation profiles in patients with chronic HBV infection.

Systemic bias occurs in microarray work due to a number of various factors. These occur at sample preparation, hybridization and measurement of methylation or gene expression. Batch to batch variation in the array manufacturing will also introduce bias during sample analysis as will the day-to-day variation in laboratory conditions that will also cause variation in results based on the day of sample preparation and analysis.

From the PCA done earlier, technical biological batch effects were found to be a big source of variation and resulted in some tissue samples clustered far from one another or hidden in technical batch effects (see **Figure 3.8A**). RMA applies quantile normalization, which is a technique that forces the distribution of the raw signal intensities of the microarray data to be the same in all the samples under analysis. This reduces the global differences being arrays but does not address the batch effect which needs to be addressed separately. After background correction and removal of batch effects through RMA, the clustering of the tissue samples became closer, easily identified and analysed (see **Figure 3.8B**).

Epigenetic mechanisms are involved in various biological processes such as the transcriptional regulation of genes and virus related tumorigenesis. Although early research showed that altered DNA methylation could silence tumour suppressor genes, the effect has also been shown to affect a wide range of genes [28 - 32]. Research continues to show that methylation of CpG island promoter regions is a key mechanism for gene silencing in many conditions [28, 209].

This study demonstrated the presence of genome wide hypermethylation of promoter CpG island regions in patients with chronic HBV infection. The possible clinical implications of such gene hypermethylation in HBV related liver and human disease are discussed (**Section 3.6.1 - 3.6.10)**. None of the gene promoter CpG islands methylated in the comparisons of HBV patients with HCC (see **Table 3.5**) and HCC with neighbouring tissues (see **Table 3.5**) were discussed since there were no

evidences that indicate that they may have relevant biological significance in the liver as far as HBV is concerned. It is also important to note that the HCC for patients used in this study was cryptogenic which means the underlying aetiology was obscure.

3.6.1 CYCLIN-DEPENDENT KINASES

The genes methylated in chronic HBV infection that are associated with cyclin-dependent kinases were *cyclin D3* (*CCND3*) and *par-6-partitioning defective 6 homolog alpha* (*PARD6A*) (see **Table 3.12.A**). These genes are implicated in various cellular signalling pathways and angiogenesis, suggesting a role in tumorigenesis. They have been reported previously as key players in the establishment of liver inflammation, fibrosis and tumorigenesis [38, 47, 48].

Protein kinases including cyclin-dependent kinases (CDK) are central regulators of the cell cycle. CDK-related protein kinases are regulated by cyclins, CDK inhibitors and phosphorylating proteins [33, 34].

Regulation of protein kinases is an important step that facilitates hepatic regeneration after injury due to viral hepatitis infections, alcohol and drugs [35 - 37]. Liver fibrosis results from a process of continuous and concurrent inflammation and healing in response to hepatocyte injury. It is accompanied by the activation of macrophage cell lines of Kupffer and other phagocytic cells, which line the liver sinusoids [38]. In response to liver damage, Kupffer cells induce inflammatory actions, activation of hepatic stellate cells and accumulation of extracellular matrix that replaces damaged hepatocytes. Importantly, genes regulating hepatic inflammatory and fibrogenic actions trigger cell cycle activation, cell growth, apoptosis and liver regeneration [38 - 44]. Inactivation of these genes by DNA methylation may disrupt wound healing and liver regeneration processes, resulting in the excessive production of extracellular matrix and severe scarring leading to cirrhosis and liver cancer.

Cyclin D3 (*CCND3*) Gene

Cyclins are targets of oncogenic viruses and play a critical role in tumorigenesis by regulating the cell cycle through their interaction with CDK-inhibitors and CDK-

related protein kinases [45, 46]. They also interact with phosphorylating proteins such as retinoblastoma and p53, whose activities are required for cell cycle progression through the G2 phase and G1/S transition [12, 47 - 49].

CCND3 protein encoded by *CCND3* gene is a prominent example of cyclins that play an important role in liver regeneration and fibrosis. Through phosphorylation of tumor suppressors, CCND3 protein regulates the activities of cyclin-dependent protein kinases CDk4 and CDk6 [47 - 49]. Activation of receptor tyrosine kinases is associated with active hepatic stellate cells (HSC) and accumulation of extracellular matrix (ECM) and therefore *CCND3* gene is important in the regulation of hepatic inflammation and fibrosis [45-49]. In HBV-induced HCC, the CCND3 protein was reported to be negatively regulated by *MiR-138* that acts as a tumour suppresser, leading to cell cycle arrest and HCC suppression [234]. In addition, high levels of CCND3 protein expression were observed in breast cancer and HBV-induced HCC with cirrhosis background [45, 46]. This correlates with loss of cell cycle control and malignant phenotypes, implying that it has a significant role in tumour progression [45, 46, 49]. In this study, there was only 1 HBV related HCC sample (see **Table 3.2**) and this would have limited the ability of the microarray analysis to show the presence of *CCND3* gene promoter hypermethylation in the comparison of HBV with HCC patients and HBV e antigen and normal controls (see **Table 3.5 to 3.7)**.

Par-6-Partitioning Defective 6 Homolog Alpha (*PARD6A*)

PARD6A is a member of the *PAR6* gene family. Mutations of *PAR6* genes cause defects during the formation of *Caenorhabditis elegans* embryo by disrupting proper asymmetric partitioning of cytoplasm [50- 52]. The *PAR6B* gene regulates the formation of tight-junctions that mediate cell to cell interaction required to maintain epithelial cell polarity and accurate asymmetric cell division processes in breast cancer cells (see **Appendix 26.G**) [51, 53].

The *PARD6A* gene is involved in asymmetrical cell division that generates two daughter cells with different cell proliferation and polarization fates [52]. It induces cell proliferation and polarization by activating atypical protein kinase C (PKC) and cell division cycle 42 (CDC42) signalling pathways [53]. The activation of PKC is

mediated by calcium ions and diacylglycerol that are produced in the cell membrane during signal transduction of inositol phospholipids [51]. HBx protein enhances diacylglycerol expression to activate the PKC signalling pathway which acts as an intracellular receptor for tumour suppressors and activators of oncogenes [54, 55].

It is possible that methylation of the *PARD6A*-associated promoter CpG island is used to alter calcium signalling as part of the virus' survival strategy. The *PARD6A* gene may also be an essential component in the biogenesis of epithelial cell tight junctions, implying that loss in the normal function of this gene may alter epithelial cell architecture and tissue remodelling leading to liver cancer.

3.6.2 GROWTH FACTORS

The CpG island promoter region of the *FGFRL1* gene was hypermethylated in HBV e antigen positive patients (see **Table 3.12.B**). The *FGFRL1* gene may be important in the development of inflammation and fibrosis through its involvement in the activation of extracellular signal-regulated protein kinases (ERK)/MAPK pathway (see **Appendix 26.J**) [69, 70]. The methylation of *FGFRL1* gene may disrupt ERK/MAPK pathway resulting in uncontrolled cell growth that could lead to malignancy.

Growth factors such as FGFRL1 encoded by *FGFRL1* gene, transforming growth factor beta (TGF-β), insulin-like growth factor (ILGF), epidermal growth factor receptor (EGFR) and tumour necrosis factor (TNF) stimulate cell growth, proliferation and apoptosis. These processes are involved in the development of hepatic steatosis, cirrhosis and HCC [56 - 58, 63, 67, 68]. They control various stages of liver regeneration by regulating inflammation, wound healing and fibrosis [59, 60]. They also control the cell cycle via the regulation of *c-Myc* proto-oncogene and Smad proteins that transduce extracellular signals from growth factor ligands to the nucleus where they regulate gene transcription.

The disruption of growth factors TGF-β, ILGF, EGFR and TNF during HBV infection has been described in human hepatoma cell lines, transgenic mice and woodchucks, resulting in increased cell proliferation, apoptosis and premalignant alteration of hepatocytes [61 - 68]. In HBV-related HCC, EGFR has been shown to be

targeted by HBx protein for cell growth [65]. In addition, HBx protein induces apoptosis by directing or sensitising the infected hepatocytes to growth factor related apoptotic pathways [65 - 68].

Par-6-Partitioning Defective 6 Homolog Alpha (PARD6A) Gene

The *PARD6A* gene regulates the signalling pathway of *transforming growth factor beta* (*TGF-β*) by activating Smad proteins and triggering the induction of epithelial-to-mesenchymal transition (EMT) [72, 73]. EMT is a potent regulator of hepatocytes migration, survival, inflammation and fibrosis, and contributes to the development of malignant invasion and metastasis ability [71 - 74]. Upregulation of the *TGF-β* gene correlates with high levels of HBx protein in the livers of transgenic mice, suggesting that the *PARD6A* gene may be involved in the transactivation of HBx protein [75].

3.6.3 METABOLIC REGULATORS

Prostaglandin reductase 2 (*PTGR2*) and *transient receptor potential cation channel subfamily v member 5* (*TRPV5*) genes were found to be hypermethylated in HBV patients (see **Table 3.12.C**). These genes are involved in lipid and glucose metabolic pathways.

Abnormal regulation of hepatic metabolism is a recently described novel feature of HBV infection and HBV-related HCC [76]. Disruption in the transcriptional activities of metabolic regulators could result in the accumulation of mutations leading to carcinogenesis [80]. A common consequence of disruption of metabolism regulators by HBx protein in the liver is hyperglycemia. Hyperglycemia is characterised by excessive glucose levels in the blood and causes liver damage that can lead to the development of fibrosis, cirrhosis and HCC [80, 81].

HBx protein disrupts metabolism by interfering with *peroxisome proliferator-activated receptor gamma* (*PPARγ*) gene, RAR-β2, inducible nitric oxide synthase (iNOS) and SREBP resulting in hepatitis steatotis and hyperglycemia (see **Appendix 26.K**) [77 - 81]. This would lead to inflammation, cirrhosis and HCC overtime [77].

Prostaglandin Reductase 2 (*PTGR2*) Gene

The *PTGR2* gene encodes PTGR2 protein that controls hepatic lipid and glucose metabolism by suppressing the expression of PPARγ protein. PTGR2 protein catalyses NADPH-dependent reduction of 15-keto-prostaglandin E2 (PGE_2) into 13,14-dihydro-15-keto-PGE_2. This catalysis suppresses PPARγ protein, a key negative regulator of hepatocyte proliferation, apoptosis, lipid and glucose metabolism [80]. NADPH is a potent ROS inducer, suggesting that *PTGR2* gene may be involved in the induction of apoptosis by HBx protein-upregulated ROS that establishes oxidative stress and hepatic injury [81].

HBx protein transcriptionally activates *PPARγ* gene via ROS upregulation and increases the expression of lipogenic and gluconeogenic genes [81]. These effects are associated with the activation of iNOS and JNK pathways resulting in significant elevation of lipid and glucose concentrations in the blood that can cause severe liver damage [80, 81] (see **Appendix 26.M)**. Abnormal metabolism of lipids and glucose impairs liver function and causes insulin resistance in type 2 diabetes mellitus, which is often seen in HBV infection and predisposes to HCC development (see **Appendix 26.M**) [80].

Another outcome of *PTGR2* hypermethylation is the disruption of the function of cylooxygenase-2. Cyclooxygenase-2 produces prostaglandins which are important in the development of tissue inflammation. It is highly expressed in most tumours including HBV-related HCC [211]. In HBV-related HCC, cyclooxygenase-2 increases the expression of HBx protein which forms a complex with p53 protein in the cytoplasm and disrupts p53-mediated transcription and apoptosis by activating prostaglandin E_2 [212].

These findings suggest that the significant methylation of the *PTGR2* gene promoter shown in this study may be one of the mechanisms utilised by HBx protein to take over control of p53-mediated apoptosis and transactivate the *PPARγ* gene to benefit viral survival. However, the adverse effects of this are abnormal lipid and glucose metabolism leading to the development of steatosis, hyperglycemia, inflammation and cirrhosis.

***Transient Receptor Potential Cation Channel Subfamily V Member 5* (*TRPV5*)**

TRPV5 gene is a member of the transient receptor family and is a fundamental cofactor of calcium and phosphorus metabolism. It is regulated by the vitamin D receptor (VDR) which is activated by 1,25-dihydroxyvitamin D3 (vitamin D). Vitamin D induces the immune system by initiating cytokine production that regulates cell growth and proliferation [82]. Low levels of vitamin D are seen in patients with liver failure and active tuberculosis as a result of impaired synthesis [83 - 86, 241]. Recent studies have shown a significant association between low serum levels of vitamin D and HBV viral loads in patients with chronic HBV infection [83, 84].

The *TRPV5* gene is an important regulator of calcium metabolism, suggesting that abnormal inactivation of TRPV5 protein expression by HBx protein-induced methylation may help to stimulate viral replication while affecting cell cycle progression, proliferation and differentiation [87, 88]. Several studies suggest that HBx protein increases cytosolic calcium uptake and stimulates HBV replication via the activation of store operated calcium entry, AP-1 and tyrosine kinase transcription pathways which influence cell proliferation and apoptosis [54, 87 - 92]. This effect may be achieved via the methylation of *TRPV5* gene.

3.6.4 TUMOUR SUPPRESSOR GENES

The tumour suppressor gene *GABPB2*, *NACC2*, *NAIF1*, *PA2G4*, *PATE*, *PCGF1*, *PARD6A*, *PTGR2*, *TBC1D17* and *TUBA4A* were hypermethylated in HBV patients (see **Table 3.12.D**). The *NACC2* and *NAIF1* genes are located in chromosome 9, which interestingly appeared to be highly methylated when HBV infected patients were compared to normal controls (see **Figure 3.10B**).. Hypermethylation of chromosome 9 in chronic HBV infection may be attributed to the know presence of a large amount of oncogenes and tumour suppressor genes located in this chromosome. These would render the, chromosome more vulnerable to HBV-induced DNA methylation[247]. In addition, it is not known whether or not HBV has preferential sites of viral integration, e.g. at sites of oncogenes and tumour suppressor genes, which would also make a chromosome with a high density of such genes more susceptible to HBV viral integration and therefore sustain more DNA hypermethylation as part

of the innate response to the presence of viral DNA in the genome.

Tumour suppressor genes play a critical role in liver carcinogenesis. They maintain various cellular pathways that regulate cell-cycle, growth, proliferation and apoptosis. When disrupted, malignant transformation and ability to invade other tissues and metastasize occurs in the affected cells [28 - 31] . Inappropriate DNA hypermethylation is an important factor in driving abnormal inactivation or silencing in the expression of tumour suppressor genes [56, 93 - 96].

GA Binding Protein Transcription Factor Beta Subunit 2 (*GABPB2)* Gene

GABPB2 gene is a member of the GA binding proteins and has been described as one of E26 transforming sequence (ETS)-associated transcription factors [97, 98]. ETS factors regulate the expression of cancer-related genes when targeted by cellular signalling pathways such as MAPK/ERK and JNK [99]. They are key players in tissue remodelling, cell growth, differentiation, apoptosis and metastases. They regulate the immediate early response genes, suggesting that *GABPB2* might be expressed at an early stage of infection [100]. Since some ETS factors regulate the production of ROS, the *GABPB2* gene may also be involved in ROS activities [101].

GABPB2 gene modulates the expression of *yes-associated protein (YAP)* oncogene which inhibits ROS-induced oxidative stress and apoptosis via the activation of the JNK pathway. In HCC, loss of YAP results in G1/S cycle arrest and increased apoptosis that drives tumorigenesis [102]. The expression of the *YAP* gene is also modulated by HBx protein via the activation of cAMP response element binding (CREB) to promote cellular growth in hepatoma cells expressing HBV-induced HCC [103]. It is possible that during the course of HBV infection, the transcriptional activity of the *GABPB2* gene is activated or silenced via HBx protein-induced aberrant methylation. This would alter the activities of CREB, c-Jun N-terminal kinase (JNK) and mitogen-activated protein kinases (MAPK)/ERK signalling pathways leading to apoptosis and compensatory hepatocyte proliferation.

NACC Family Member 2, BEN and BTB (*POZ*) *Domain Containing* (*NACC2*) Gene

NACC2 is a novel gene. Little is known about the regulation and physiological relevance of this gene in viral infections and cancers. The *NACC2* gene has been linked to the negative regulation of the mitochondrial intrinsic apoptotic signalling pathway, which may be disrupted by HBx protein to increase viral replication [105]. The intrinsic signalling pathway is characterised by the release of the cytochrome C into the cytoplasm where it induces apoptosis by increasing caspase 3 activities [104].

It is possible that in response to damage caused by HBV infection, the *NACC2* gene controls apoptosis by regulating the intrinsic pathway via its interaction with p53 transcriptional activator or cytosolic cytochrome C. A study by Lee et al[105] showed an increase in the level of cytosolic cytochrome C in HeLa and HepG2 cells-expressing HBx protein. Hypermethylation of the *NACC2* promoter CpG island may be a mechanism by which the HBx protein causes the release of cytochrome C for apoptosis induction in infected hepatocytes.

Nuclear Apoptosis Inducing Factor 1 (*NAIF1*)

The *NAIF1* gene, also known as *Chromosome 9 open reading frame 90* (*C9orf90*), encodes the nuclear localised NAIF1 protein that is highly expressed in liver and gastric tissues [106]. Expression of the NAIF1 protein in oesophageal carcinoma and gastric cancer cells regulates cell proliferation and apoptosis by activating caspases 3, 8 and 9 pathways. Downregulation of NAIF1 protein expression was shown to induce cell cycle arrest (G0/G1) and apoptosis in gastric cancer cells [106]. Since caspases 3 and 9 regulate apoptosis [244], methylation of promoter region CpG island in the *NAIF1* gene may be another mechanism by which HBx protein alters caspases 3 and 9 pathways leading to induction or inhibition of apoptosis that is associated with increased viral replication and cell survival.

Par-6-Partitioning Defective 6 Homolog Alpha Gene

The *PARD6A* gene induces the expression of the *TGF-β* gene, which displays its tumour suppressive role by inhibiting cell proliferation and inducing apoptosis in

cultured hepatocytes (see **Appendix 26.B**) [107, 108]. Expression of the TGF-β protein in liver cells is associated with a highly invasive phenotype accompanied by recurrence of aggressive tumours and metastasis [109]. HBx protein deregulates phosphatidylinositol 3 kinase (PI3K) and phosphatase and tensin homolog (PTEN) pathways in order to inhibit the *TGF-β* induced apoptosis in hepatoma cells [110, 111]. The ability of the *PARD6A* gene in regulating apoptosis might be HBx protein-dependent and therefore may play a critical role in the pathogenesis HBV infection and development of HBV-induced HCC.

Proliferation Associated 2G4 (*PA2G4*) Gene

The *PA2G4* gene encodes PA2G4 protein that is also known as V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (ErbB3)-binding protein 1 (EBP1) [112]. The PA2G4 protein (EBP1) is known to inhibit the expression of E2F1 and androgen receptor-regulated genes such as prostate specific antigen by binding to and activating Sin3A-histone deacetylases (HDAC) 2 complex [221, 222]. Sin3A-HDAC2 complex repress the transcription of the targeted gene by binding to its promoter. Repression of E2F1 and AR mediated transcription inhibits proliferation and induces differentiation in human breast and prostate cancer cell lines [114, 221 -223]. The downregulation of the PA2G4 protein in leukemia, breast, bladder and prostate cancer cells is associated with increased cell growth, proliferation, differentiation and tumour progression [95].

The PA2G4 protein also interacts with the cytoplasmic domain of inactive tyrosine kinase ErbB3 protein [112]**.** The ErbB3 protein is an active receptor of ligand heregulin that stimulates its tyrosine phosphorylation and enables it to dissociate and translocate to the nucleus [224]. This induces intracellular adhesion molecule 1 expression leading to cellular proliferation and differentiation [225, 226]. Apart from ErbB3 protein, the *ErbB3* gene also encodes an epidermal growth factor receptor (EGFR) which activates the PI3K-AKT and JNK-MAPK pathways, which are involved in DNA synthesis, cell proliferation and apoptosis. Their disruption will contribute to malignant transformation of liver cells [113].

The PA2G4 protein is a cell cycle regulatory DNA-binding protein. It appears in the

nuclei from late G1 to early S phase and disappear late at G2, suggesting its important role on cell cycle progression [227]. Mouse recombinant PA2G4 protein was shown to have an inhibitory effect on DNA replication in *Xenopus* egg extracted single stranded DNA template [114]. Methylation and disruption of the *PA2G4* gene in chronic HBV infection would result in inhibited apoptosis leading to uncontrolled cell growth, differentiation and abnormal DNA synthesis.

Prostate and Testis Expressed 3 (*PATE3*) Gene

The *PATE3* gene encodes the ataxia telangiectasia mutated (*ATM*) gene and is abundantly expressed in the prostate and testis. Mutations in the *ATM* gene result in predisposition to infections, disrupts cell cycle arrest, DNA repair and apoptosis via phosphorylation of the *p53*, *checkpoint kinase 2* (*CHK2*) and *Histone 2A member X* (*H2AX*) tumour suppressor genes [115]. In human glioma cells, knockdown of the *ATM* mutated gene was shown to trigger DNA damage by increasing the level of ROS and altering radiation-induced apoptosis [116, 117].

Methylation of the *PATE3* gene during HBV infection may either inactivate or upregulate the level of mutated *ATM* gene, altering the cellular p53 and ROS signalling pathways which then worsen DNA damage caused by HBV infection.

Polycomb Group Ring Finger 1 (PCGF1) Gene

The *PCGF1* gene is a mammalian homolog of the Drosophila polycomb genes that negatively regulates the cell cycle via the activation of B-cell CLL/lymphoma (Bcl)-6 and p21 cyclin-dependent kinase inhibitor [118]. The *Polycomb* genes typically modify histone tails by reversibly repressing the transcriptional activity of genes specific to cell fate thereby influencing hepatocyte transformation [119]. The transcriptional repressive capability of the *PCGF1* gene, its interaction with the *Bcl-6/p21* genes and association with enhanced cell cycle progression and proliferation suggests a possible role of the *PCGF1* gene in tumorigenesis.

The *PCGF1* gene encodes a protein that contains the RING finger motif conserved with cysteine rich domain which is involved in the monoubiquitination and

degradation pathway of histone H2A protein [118, 120]. The expression of the *p21* gene depends on the p53 signalling pathway, suggesting that the *PCGF1* gene may induce histone H2A monoubiquitination by disrupting the p53 signalling pathway. The *PCGF1*- induced monoubiquitination switches off the transcriptional elongation of RNA polymerase II and this suggests that it also has a role in gene transcription [120]. Although there is no known direct association of the *PCGF1* gene with HBV infection, alteration in the expression of encoded PCGF1 protein may be involved in carcinogenesis through abnormal ubiquitination and gene silencing.

Prostate Cancer Overexpressed 1 (*PTOV1*) Gene

The *PTOV1* gene encodes a novel mitogenic protein that is expressed at a high level in malignancies [122]. The PTOV1 protein acts as a tumour suppressor and potential marker for cancer progression due to its increased level of expression and enhanced cell proliferation in high-grade bladder and prostate cancers [122]. The PTOV1 protein shuttles dynamically from the cytoplasm into the nucleus to promote the entry into the S phase of the cell cycle. *Insulin-like growth factor-1* (*IGF1*) is a known inducer of cell cycle S phase entry-related gene that upregulates the phosphorylation of retinoic acid-induced retinoblastoma contributing to G0/G1-S transition [121]. *IGF-1* promotes cell cycle progression via activation of the PI3K and NF-κB signalling pathways that are regulated by ROS and implicated in the transactivation of HBx protein [122].

In the lung cancer, the interaction of the *PTOV1* gene with *Zyxin*, a nuclear-translocated gene that regulates cell adhesion and motility occurs through the activation of transforming growth factor beta (TGF-β)/Smad3 [46]. The collaboration of the *PTOV1* and *Zyxin* genes was shown to negatively regulate the *retinoic acid* promoter region by suppressing of retinoic acid receptor activities, leading to enhanced cell growth and proliferation [123]. Though the underlying biological mechanisms related to the *PTOV1* gene in HBV infection have not yet been elucidated, it is possible that promoter methylation of the *PTOV1* gene serves as a key silencer of gene transcription that is important in the development of HBV related liver disease and HCC.

Prostaglandin Reductase 2 (*PTGR2*) Gene

The *PTGR2* gene is highly expressed in the liver and is required for activating the CREB pathway which is involved in liver regeneration and disrupted in malignancy [124]. CREB is a cellular transcription factor that binds to the cAMP response element (CRE) in the promoter regions of the target genes thereby limiting or inducing their transcription [124].

The loss of PTGR2 protein expression in gastric cancer cells is associated with altered mitochondrial function that leads to increased ROS production and apoptosis [125]. In addition, the ERK1/2 and caspase 3 pathways that usually co-operate with the CREB in producing ROS are activated and lead to gastric cancer progression accompanied by increased expression of the Bcl-2 protein [126]. The Bcl-2 protein typically maintains induced mitochondrial oxygen consumption [125].

Increased expression of the PPARγ ligand, a binding partner for CREB, has been shown to activate hepatic stellate cells leading to fibrosis associated with cell cycle arrest, growth and increased proliferation in tumour cells [127, 128]. CREB is transactivated by HBx protein causing increased cell survival and is also activated by methyl groups thereby inhibiting gene transcription [129, 131]. These findings suggest that the *PTGR2* gene may be methylated and transcriptionally altered in HBV infection resulting in the activation of the CREB pathway to facilitate the survival of infected hepatocytes.

TBC (*tre-2/USP6, BUB2, cdc16*) *1 Domain Family, Member 17* (*TBC1D17*) Gene

The *TBC1D17* gene encodes three protein domains including Ubiquitin specific protease 6 (USP6)/Tre-2 oncogene (tre-2), Budding uninhibited by Benzinidazole 2 (BUB2) and Cell division cycle 16 (CDC16). The *TBC1D17* gene regulates cell transformation, proliferation and cytokinesis by binding to the auxin response factors (ARF)-GTPase activating protein (ArfGAP) [132]. This binding stimulates the transcriptional activities of NF-κB, mammalian target of rapamycin (mTOR) and Wnt/β-catenin signalling pathways required for liver regeneration [233].

In HBV-induced HCC, there is upregulation of the mTOR signalling pathway by HBx protein via NF-κB pathway associated with inhibition of cell transformation and

proliferation [133]. An mTOR-dependent signalling pathway leads to activation of hepatic stellate cells, which in turn activate leucine residues of hepatocyte growth factor, stimulating the production of hepatocyte growth factor [245]. Hepatocyte growth factor is essential in tissue regeneration, angiogenesis and tumorigenesis due to its ability to stimulate mitogenesis, cell motility and matrix invasion. When abnormally regulated, hepatocyte growth factor may lead to tumorigenesis [245]. Hypermethylation of *TBC1D17* gene may disrupt mTOR and NF-κB signalling pathways and affect the physiological activities of hepatocyte growth factor, leading to increased cell proliferation and malignant transformation in the liver.

Tubulin Alpha 4A (*TUBA4A*) Gene

The *TUBA4A* gene is a microtubule associated oncogene that maintains the cell structure and is required for cell adhesion [134]. The *TUBA4A* gene is a regulator of the Wnt/β-catenin pathway, which initiates transcription by binding to T-cell factor (TCF)/Lymphoid enhancing factor (LEF) transcription factors through its β-catenin binding region. The Wnt/β-catenin pathway regulates cell-cell adhesion and this may explain its association with the *TUBA4A* gene [135]. Microtubule proteins often increase their stabilization by interacting with the *APC* tumour suppressor, which inhibits uncontrolled cell proliferation by suppressing the activity of Wnt/β-catenin pathway. Downregulation of the APC protein was shown to increase Wnt/β-catenin transcriptional activity in a mouse model, resulting in increased cell proliferation, deregulated protein metabolism, hepatomegaly and ultimately HCC development [135].

The APC protein forms a complex with binding partners and destroys β-catenin via ubiquitin-independent degradation. This process is important to inhibit cell proliferation by repressing genes that induce cell division and growth from being switched on more than is necessary. The HBx protein competitively interacts with APC and displaces β-catenin from APC-mediated ubiquitination and degradation [136]. This upregulates the Wnt/β-catenin signalling pathway causing uncontrolled cell growth that would favour the development of HCC. It is possible that the *TUBA4A* gene is upregulated via HBV-induced hypermethylation allowing HBx protein to alter the Wnt/β-catenin signalling pathway, disrupt ubiquitination and increase the risk of malignancy.

3.6.5 TRANSCRIPTION REGULATORS

RNA polymerase 1 transcription factor homolog pseudogene 3 and *GA binding protein transcription factor beta subunit 2* genes were hypermethylated in HBV infection and are transcriptional regulators (see **Table 3.12.E**).

HBx protein may also contribute to liver tumorigenesis by deregulating RNA and DNA dependent transcription factors [137, 138]. HBx protein binds to core promoter TATA binding proteins (TBPs) and TBP-associated factors (TAFs) that normally form the transcription factor II human (TFIIH) complex [139 - 142]. The TBP and TFIIH complex are p53-dependent basal transcription factors and are required for normal transcription initiation, cell-cycle control, DNA excision and repair processes [140, 143, 148].

HBx protein disrupts the p53 pathway by binding to the carboxy terminus of TBP and TFIIH transcription factors [140]. This leads to inhibition of DNA repair causing DNA damage and accumulation of mutations that activate tumour invasion and metastasis properties in cells [141, 143, 153 - 155].

The TBP and TFIIH complex encodes the TATA box-binding protein-associated factor RNA polymerase I subunit B (TAF1B) protein and mutations in its gene is associated with colorectal cancer microsatellite instability, characterised by the presence of multiple copies of short tandem DNA repeat sequences [149,152].

RNA Polymerase 1 Transcription Factor Homolog Pseudogene 3 (*RRN3P3*) Gene

The *RRN3P3* gene is transcribed by RNA polymerase 1, which is also involved in the synthesis of ribosomal RNA genes [228]. Although there is not much information about this gene and human diseases including cancer, *RRN3P3* gene may play a role in HBV infection as the HBx protein has been described as a novel virus transcriptional modulator of RNA polymerases and TFIIB [228]. HBx protein specifically binds to RNA polymerase II subunit B, which when highly expressed stimulates the transcription of HBx protein [228]. Hypermethylation of *RRN3P3* gene (see **Table 3.12.E**) may be a strategy for viral infection to increase the transcription of HBx protein.

GA Binding Protein Transcription Factor Beta Subunit 2 (GABPB2) Gene

GA binding protein (GABP) transcription factor activates genes required for DNA methylation and histone modification contributing to self-renewal, tissue regeneration and differentiation [157 - 159]. GABP induces growth and antioxidant defence by activating Hippo pathway [50].

GABPB2 transcription factor, encoded by *GABPB2* gene, is a member of GABP family proteins. It contains ankyrin repeats in its promoter and regulates transcription by forming a tetrameric complex with the DNA-binding alpha subunits. It also has transcriptional activation domain (TAD) that mediates the initiation and elongation of RNA polymerase II transcription [156].

Hypermethylation of the *GABPB2* gene promoter CpG island may result in the silencing of *GABPB2* gene expression. This could interfere with normal regulation and function of this gene leading to continuous hepatic damage, cirrhosis and HCC.

3.6.6 REGULATION OF RNA SPLICING

Microarray analysis revealed hypermethylation of CpG islands in the promoter regions of a number of the RNA processing-related genes that may be implicated in the transcriptional activities of the HBx protein (see **Table 3.12.F**).

The DNA methylation machinery has been described in transposons and other DNA repeats where it alters the regulation of mRNA splicing, which is important for proper protein folding and mRNA production. In eukaryotes, mRNA sequences with introns and exons are transcribed from DNA. The pre-mRNA transcripts undergo post-translational modifications which excise introns from the sequence by splicing and joining the exons to form a contiguous coding sequence [176].

Disruption of the mRNA splicing pattern often results in incomplete intron excision that leads to loss of protein expression or aberrant protein production. About 60% of disease-causing mutations are associated with abnormal mRNA splicing [176]. Accumulation of abnormal or aberrant mRNA splicing has been identified mostly in cancerous cells and is rare in normal cells, suggesting that abnormal mRNA splicing contributes to tumorigenesis [177].

In HBV infection, the HBx protein activates transcription factors that stimulate the expression of RNA processing-related genes through its interaction with *cis*-acting elements [178 - 180]. Regulation of RNA processing-related genes may play an important role in the pathogenesis of HBV infection.

Exosome Component 3 (*EXOSC3*) Gene

The *EXOSC3* gene encodes the ribosomal RNA–processing protein 40 (Rrp40), which is characterised by having 5' to 3' exoribonucleolytic activity. The 5' to 3' exoribonucleolytic activity of Rrp40 facilitates the degradation of the EXOSC3 protein. The cytoplasmic exosome complex is involved in the normal turnover and degradation of mRNA that has been tagged for degradation via nonsense-mediated RNA decay (see **Appendix 26.H**). This mRNA surveillance pathway is required for the elimination of mRNA transcripts that contain errors and have premature stop codons [181].

Non-stop decay is another mRNA surveillance pathway and is required for the detection of mRNA molecules that lack stop codons and prevents them from being translated. It blocks the translation of such mRNA molecules by directing them towards the nuclear exosome complex for degradation (see **Appendix 26.H**) [182].

Given these critical processes, it is evident that hypermethylation of the *EXOSC3* gene promoter CpG island may alter the transcriptional activities of Rrp40 leading to abnormal regulation of these critical pathways. Consequently, this may cause the accumulation of aberrant mRNA transcripts that, if translated, will either result in the loss of anticipated protein production or antimorphs which are dominant negative mutations that antagonise normal gene activity [183].

Small Nucleolar RNA C/D Box 48 (SNORD48) Gene

The *SNORD48*, also known as *chromosome 6 open reading frame 48* (*C6orf48)* is a relatively newly described gene that belongs to a class of small nucleolar RNA (snoRNAs) molecules. The SnoRNA genes with C/D box motif play a part in the normal 2'-O-ribose methylation of rRNA, small nuclear RNA (snRNA) and transfer

RNA (tRNA). Induction of the snoRNA methylation by *SNORD48* gene is important for stability, proper snoRNA processing and nucleolar localization [184]. Such genes are frequently downregulated in cancer cells as they are located at the chromosomal fragile sites that are in close proximity to tumour suppressor genes [185].

Chromosomal fragile sites in HBV infection have been described by Murakami et al[186] who used by Alu-PCR based techniques to show that the HBV genome integrates at chromosomal fragile sites in human chromosomes. HBV genome integration at chromosomal fragile sites was shown to lead to chromosomal instability resulting in the accumulation of mutations and epigenetic alterations [186].

Hypermethylation of the promoter regions of genes similar to the *SNORD48* gene were also identified in this study and these include the *small nucleolar RNA H/ACA box 52 (SNORA52)* and *small nucleolar RNA H/ACA box 7D (SNORA7D)* genes (see **Table 3.7** and **3.11**).

Small Nucleolar RNA H/ACA Box 52 (SNORA52) and *Small Nucleolar RNA H/ACA Box 7D (SNORA7D)* Gene

Compared to the *SNORD48* gene, the *SNORA52* and *SNORA7D* genes have a H/ACA box motif that aids in guiding pseudouridylation which is the conversion of uridine to pseudouridine [185]. Induction of pseudouridylation by ROS-related oxidative stress has been reported suggesting that the *SNORD48* gene is involved in regulating apoptosis through the transactivation role of the HBx protein [185]. It is possible that these genes are involved in the induction of ROS-related oxidative stress by the HBx protein.

Thyroid Hormone Receptor Associated Protein 3 Gene

Another mRNA splicing-related gene is the *THRAP3* which is also a VDR-regulated gene. The *THRAP3* gene is also known and reported as *TRAP150* [187]. The *THRAP3* (*TRAP150*) gene regulates mRNA splicing and initiates thyroid hormone associated-gene transcription via activation of cell signalling pathways that involve transcription and initiation factors. The *THRAP3* gene (*TRAP150*) may exert its transcription

activating role by binding to the Transcription elongation factor 1 (TCERG1/CA150) [188]. The *TCERG1* gene contains two conserved F residues (FF) and WW domains that mediate protein-protein interaction. It uses these domains to interact with RNA polymerase II and the pre-mRNA splicing factor SF1 [188, 189]. There is no documented link between *THRAP3* gene and liver disease. However, it is possible that HBx protein forms a complex with *THRAP3* gene and disrupts protein-to-protein interactions between *THRAP3* and transcription factors such as TCERG1. This would prevent the transcriptional transactivating properties of *THRAP3* gene leading abnormal mRNA splicing that would influence the progression of liver disease.

The *TCERG1* gene is involved in the regulation of the HIV-1 Tat protein expression [190]. Overexpression of TCERG1 protein is associated with significant reduction of Tat-dependent activation of HIV-1 Long terminal repeat (LTR) promoter and transcription initiation *in vivo* [190]. This may explain the interaction of the *THRAP3* gene with HIV-1 activated proteins in human cell lines [191]. In the presence of HBV and HIV coinfection, the potential interaction would result in the suppression of TCERG1 protein due to HBV related methylation. This would activate HIV-1 LTR promoter, leading to increased expression of Tat protein and enhanced viral replication.

3.6.7 VITAMIN D RECEPTOR REGULATED GENES

Hypermethylation of the promoter regions of the VDR-related genes *transient receptor potential cation channel subfamily v member 5* (*TRPV5*) and *thyroid hormone receptor associated protein 3* (*THRAP3*) were noted in patients with chronic HBV infection (see **Table 3.12.G**).

Vitamin D receptor (VDR) is a ligand activated transcriptional regulator of calcium absorption [163, 164]. It usually forms a heterodimer complex with retinoid x receptor to cause gene transcription [229]. It also causes gene transcription by binding to 1,25-dihydroxyvitamin D_3 via vitamin-D responsive elements (VDRE) of VDR-targeted genes which stimulates cell-mediated immunity and may inhibit cell proliferation [230 - 232].

HBx protein stimulates viral replication and core viral particle assembly by increasing the mitochondrial calcium signals in HepG2 cells expressing HBx protein. This induces cell proliferation and favours tumorigenesis. Recent studies show that the level of 25-hydroxyvitamin D is very low in the presence of chronic HBV infection, suggesting that vitamin D deficiency may play a role in enhancing viral replication [83, 84]. The binding of vitamin D to the BH3 binding motif of Bcl-2 related proteins controls the production of various proteins involved in calcium transport and consumption [54, 87, 168]. High calcium levels caused by the HBx protein control the cells and induce apoptosis via activation of transcription factors such as the Bcl-2 and AP-1 proteins [168, 169]. It is possible that hypermethylation of the VDR-related promoter genes is a key mechanism through which HBx protein binds to Bcl-2 related proteins and modulates calcium signalling and increases the replication of HBV.

VDR is a highly polymorphic and is implicated in various malignancies such as breast, colorectal and cervical cancer [160]. Some studies have established an association between VDR polymorphisms and the outcome of HBV infection in an Asian population from China and Taiwan. This correlation is thought to influence the genetic susceptibility to HBV infection in these populations [161, 162].

Transient Receptor Potential Cation Channel Subfamily V Member 5 Gene

The *TRPV5* gene is transcriptionally regulated by VDR and parathyroid hormone (PTH) via protein kinase A and C. Its main role is to maintain the serum level of calcium and phosphorus in the blood. It interacts with S100 calcium binding proteins and vitamin D-dependent protein containing EF-hand calcium binding motifs. Activation of the S100 proteins by vitamin D receptors regulates cell cycle progression, proliferation and differentiation [170]. In response to low calcium, parathyroid gland secretes PTH to raise calcium levels in the blood by increasing renal calcium reabsorption [171].

Mouse studies showed that the *TRPV5* gene mediates renal calcium reabsorption and its downregulation suppresses this process [172]. Loss or upregulation of the *TRPV5* gene expression from HBV-induced hypermethylation may result in the same

conditions associated with the VDR mutations and this may display a gateway mechanism for HBx protein to control the infected cells.

Thyroid Hormone Receptor Associated Protein 3 (*THRAP3*) Gene

The *THRAP3* gene forms a thyroid hormone receptor-associated protein (TRAP) complex by interacting with thyroid and steroid receptors [173]. This interaction facilitates ligand-dependent binding of TRAP factors to nuclear receptors that regulate cell cycle, growth and differentiation [173]. The *THRAP3* gene is involved in vitamin D receptor binding, suggesting that it may also be involved in the regulation of intracellular calcium uptake and utilisation. The cytochrome P450 (CYP) superfamily is a large group of enzymes abundantly expressed in the liver and involved in the metabolism of oxidative molecules by converting them into water-soluble molecules for clearance [173].

It has been shown that the VDR activates CYP family 27 subfamily B polypeptide 1 (CYP27B1) protein that regulates 1,25-dihydroxyvitamin D_3 and causes induction of cathelicidin and β-defensin antimicrobial peptides that act as innate immune defenses against infection [174, 175]**.** Hormonal imbalance resulting from these events may result in reduced ability to mount effective innate immune responses against HBV infection, abnormal cell proliferation and apoptosis with an increased risk of malignancies [171]. Alteration in the transcriptional activity of the *THRAP3* gene via HBV-induced hypermethylation may negatively regulate VDR binding and may reduce cathelicidin and β-defensin induction by suppressing CYP24A1. This may also help to explain the low levels of vitamin D seen in Asian patients with HBV infection [83].

3.6.8 GENES ENCODING UBIQUITIN PROTEINS

In this study, HBV patients had hypermethylation of four genes that encode ubiquitin proteins. These were *UBX domain protein 1* (*UBXN1*), *ligand of Numb protein X 2* (*LNX2*), *PA2G4* and *thyroid hormone receptor interactor 12* (*TRIP12*) genes (see **Table 3.12H**).

Most ubiquitin-regulated genes are tumour suppressor genes that are involved in the development of cancer by regulating signal transduction, cell cycle arrest and proliferation [192]. Disruption of the normal ubiquitination activities of these genes may alter p53, Notch and Wnt cellular signalling pathways contributing to tumour initiation and progression [193, 194]. Methylation of such genes is rare in normal tissue or epithelial cells but is frequently present in malignant cells, suggesting that they could potentially be used as tumour markers for the early detection of many malignancies including HCC [195, 199].

The HBx protein binds ubiquitin proteins to protect itself from degradation [201]. It is possible that the HBx protein disrupts the normal physiological functions of ubiquitin proteins to achieve its transactivation role leading to increased viral replication and protection against immune defence mechanism [200, 201]. The reported mechanisms underlying the regulation of ubiquitin proteins and their physiological role in HBV or HBV-induced HCC are still not fully elucidated.

UBX Domain Protein 1 (UBXN1) Gene

The UBXN1 is a cellular receptor protein that interacts with the cell cycle regulator BRCA1-associated RING domain protein 1 (BARD1) heterodimer and regulates its activity in an ubiguitin-independent manner. This interaction triggers a critical block in the enzymatic functions of the BARD1 protein which involves E3 ubiquitin ligase activity. Elevated levels of *UBXN1* are associated with a significant reduction of E3 ubiquitin ligase activity. Many cases of ovarian and breast cancer have germline mutations of *BRCA1* gene [202]. Amplified in breast cancer 1 (AIB1) protein is highly expressed in human HBx protein positive HCC tissues, but rarely in HBx protein negative HCC tissues and this correlates with enhanced cell proliferation and HCC progression [203]. Hypermethylation of the *UBXN1* promoter CpG island during HBV infection may be another mechanism by which HBx protein causes gene transactivation.

Ligand of Numb protein X 2 (*LNX2*) Gene

The *LNX2* gene is a member of the Ligand of Numb protein X family proteins that are implicated in the development of the nervous system in Drosophila [204]. The *LNX2* gene has PDZ-domain containing RING finger motif that acts as the E3 ubiquitin ligase which binds to phosphotyrosine domain of Numb and promotes its degradation via the ubiquitin-proteasome pathway[204]. The LNX proteins are the key regulators of cell fate decisions, differentiation and proliferation, suggesting their role in cancer[192].

In breast cancer, loss of LNX2-activated Numb protein activates of the oncogene Notch signalling pathway and suppresses p53 activity [204]. Furthermore, overexpression of the LNX2 protein activates Notch signalling and ultimately Wnt/β-catenin pathway in colorectal cancer [204]. A recent study by Luo et al[205] suggests that the HBx protein downregulates the NF-κB transcriptional factor by reducing the activity of the Notch signalling pathway and contributes to liver tumorigenesis. The p53, Wnt/β-catenin and NF-κB signalling pathways have been shown to be the central targets of HBx protein [205], suggesting that any alteration in either the *LNX2* or Numb gene may also be important in HBV liver disease pathogenesis and cancer development.

Proliferation Associated 2G4 (*PA2G4*) Gene

Apart from being a tumour suppressor gene, the *PA2G4* gene may be oncogenic in cells by interacting with human double minute-2 protein (HDM-2) via the ErbB3 binding isoform [206]. The HDM-2 protein is an E3 ubiquitin ligase that, when overexpressed, inhibits p53-mediated cell cycle arrest and apoptosis by suppressing the p53 signalling pathway [207]. Given this data, hypermethylation of the *PA2G4* promoter CpG island in HBV infection cause the overexpression of PA2G4 protein, leading to uncontrolled cell differentiation and abnormal ubiquitination that develop into cancer.

***Thyroid Hormone Receptor Interactor 12* (*TRIP12*) Gene**

The *TRIP12* is an important ubiquitin gene and has a homologous to E6-AP carboxyl terminal - like domain that allows it to function as an E3 ubiquitin ligase that is required for DNA repair [207]. The *TRIP12* gene may play a critical role in liver disease and tumorigenesis as it is important in repairing and cleaving damaged DNA by recruiting histone 2A protein that mediates ubiquitination to the sites of damage [207].

Abnormal transcriptional activity of the *TRIP12* gene due to HBV induced methylation may either be associated with loss of the TRIP12 protein expression that will limit ubiquitination by histone 2A protein at the sites of DNA damage. This can also lead to overexpression of the TRIP12 protein that will increase the level of histone 2A protein production and lead to hyper-accumulation of ubiquitination. These events would ultimately cause mutations that can cause cancer [207].

3.6.9 PSEUDOGENES

Hypermethylation in the promoter CpG islands of pseudogenes such as *RNA polymerase 1 transcription factor homolog pseudogene 3* (*RRN3P3*), *coiled-coil domain containing 144B pseudogene* (*CCDC144B*), *defensin theta 1 pseudogene* (*DEFT1P*), *family with sequence similarity 106 member C pseudogene* (*FAM106C*) and *keratin 42 pseudogene* (*KRT42P*) were identified in patients with chronic HBV infection (see **Tables 3.4, 3.5** and **3.12.I**).

Pseudogenes have recently been defined as the newly discovered regulators of gene expression. They are ubiquitous and abundant in tumour tissues as compared to normal tissue, suggesting that they may have a role in the pathogenesis of human diseases and cancers [144]. They resemble the structure and sequence of their parental genes, but have defects that make them produce abnormal mRNA sequences that encode non-functional proteins [145, 146]. When transcribed, pseudogenes induce a series of genetic alterations such as loss of promoter sequences, frameshift mutations, deletions and insertions, giving them entirely different functions to their parental genes [147].

The clinical relevance of the hypermethylation of promoter CpG islands of *RRN3P3, CCDC144B, DEFT1P*, *FAM106C, KRT42P* pseudogenes may be the loss of normal gene expression as well as the abnormal activation of genes that could cause liver disease and malignancy.

3.6.10 GENES THAT INTERACT WITH HIV-1 PROTEINS

TUBA4A and *THRAP3* are two genes that interact with human immunodeficiency virus type 1 (HIV-1) Gag, Rev and Tat proteins which were found to be hypermethylated (see **Table 3.12.J**). *TUBA4A* gene interacts with HIV-1 Gag and Protease proteins while *THRAP3* gene interacts with Rev and Tat proteins [190, 191, 213, 216].

It is estimated that 5 - 10 % of chronic HBV infected individuals worldwide are co-infected with HIV-1 [214, 215]. HIV-1 influences HBV pathogenesis and accelerates the risk of hepatic cirrhosis and HCC. The liver related mortality is 2 -3 times higher in HBV co-infected patients compared to those with HIV-1 monoinfection [215]. The mechanisms underlying HBV and HIV-1 co-infection are still under investigation.

Tubulin Alpha 4A Gene

The *TUBA4A* gene interacts with the human immunodeficiency virus type 1 (HIV-1) Rev and transactivator of transcription (Tat) protein which is abundantly expressed in the liver [213, 216]. The Tat protein enhances the carcinogenic effects of HBV in the liver of transgenic mice through its interaction with HBx protein. It appears that interaction of *TUBA4A* gene with HIV-1 proteins depolymerises microtubules that are formed by tubulins leading to the alteration of microtubules dynamics and activation of *Bcl-2* mediated apoptosis. It is possible that HBx protein may disrupt *Bcl-2* mediated apoptosis and other apoptotic related pathways such as MAPK and Wnt signalling pathways by methylating the gene promoter *TUBA4A* region (see **Appendix 26.O**) [213].

Thyroid Hormone Receptor Associated Protein 3 (*THRAP3*) Gene

Overexpression of TCERG1 protein is associated with a significant reduction of Tat-dependent activation of HIV-1 Long terminal repeat (LTR) promoter and transcription initiation *in vivo* [190]. This may explain the interaction of the *THRAP3* gene with HIV-1 activated proteins in human cell lines [191]. It is possible that the *THRAP3* gene is expressed in the presence of high viral replication to inhibit viral DNA replication and core viral particle assembly by altering the transcriptional activity of the HBx protein. In return, HBV could trigger a self-defence mechanism by using HBx protein to inactivate the transcriptional activity of the THRAP3 protein via hypermethylation of its promoter and therefore silencing the *THRAP3* gene. This disruption would also favour the replication of HIV-1, helping to explain the severity of HBV-HIV coinfection related liver disease.

3.7 CONCLUSIONS

This study successfully demonstrates the presence of genome-wide promoter region CpG islands methylation in patients with chronic HBV infection. Hypermethylation of promoter CpG island regions of several genes was identified. These include cyclin-dependent kinases, growth factors, metabolic regulators, tumour suppressors, transcription regulators, RNA splicing regulators, ubiquitin related proteins, pseudogenes and VDR-regulated genes.

Cyclin-dependent kinases, growth factors and some tumour suppressors such as *CCND3*, *PARD6A*, *PA2G4*, *PTGR2* and *TBCD17* genes are associated with cellular signalling pathways such as activation of transcription factors TGFβ, NF-kβ, CREB, ROS, MAPK and JNK whose targets are inflammation and fibrogenic related genes (see **Sections 3.6.1**, **3.6.2** and **3.6.4**). This suggests that any alteration of their functions may result in HBV induced inflammation, fibrosis and cancer in the liver.

An important role of HBV in calcium signalling pathway was suggested by vitamin D receptor (VDR)-regulated genes including *THRAP3* and *TRPV5* (see **Section 3.6.8**), which regulates calcium homoeostasis. HBV related disruption of calcium signalling by methylation of VDR related genes has an influence on apoptosis, cancer and particularly viral replication.

Alteration of mRNA splicing in HBV infection was also suggested by the hypermethylation of the mRNA processing related genes *EXOSC3*, *SNORD48*, *SNORA7D* and *SNORA52* (see **Section 3.6.6**), which are required for proper folding of mRNA and protein production. Their abnormal function could cause abnormal mRNA splicing and subsequently abnormal protein production and function. These abnormalities would disrupt normal liver function.

Hypermethylation of genes encoding LNX2, UBXN1 and TRIP12 (see **Section 3.6.H)** ubiquitin-related proteins, supports the evidence that HBV alter the proteasomal activity of infected cells to facilitate and enhance viral replication and survival [236 - 240].

Many tumour suppressor genes were found to be hypermethylated (see **Section 3.6.4**). The evidence links this to the action of HBx protein which would increase cell survival and the risk of cancer development.

None of the identified gene promoters have previously been investigated in chronic HBV infection and related HCC. However, their cellular binding partners are known to be activated by HBx protein, which appears to be the driving force behind HBV induced gene promoter methylation and the accompanying abnormal gene function. HBx protein is an important transcriptional transactivator protein that influences viral replication and cell survival by disrupting cellular signalling pathways [96]. The hypermethylated gene promoters identified in the study are potential and known targets of HBx protein and their abnormal function would benefit the virus and its survival.

Hypermethylation is typically associated with the downregulation of gene expression [209] and therefore it is most likely that hypermethylation of the identified genes leads to downregulation of gene expression. Further studies of the gene expression profile of these hypermethylated genes will broaden our understanding of some of the gene transcriptional changes that contribute to the development of HBV induced liver injury and cancer. In addition, any such new insights may contribute to the development of new treatments and early diagnostic tools for chronic HBV infection and related HCC in sub-Saharan Africa.

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CHAPTER 4

VALIDATION OF MICROARRAY DATA FINDINGS USING BISULFITE DNA SEQUENCING

4. INTRODUCTION

Bisulfite DNA sequencing was first introduced by Frommer et al[1] as a genomic tool for mapping methylated cytosines in the early 1990s. This technique is considered to be the gold-standard for DNA methylation analysis at a single base-pair resolution. It involves sodium bisulfite conversion that differentiates non-methylated from methylated cytosines by converting methylated cytosines to uracils that appear as thymine in the bisulfite DNA converted sequence [1, 2].

Other quantitative methylation techniques include combined bisulfite restriction analysis (COBRA), methylation-sensitive single nucleotide primer extension (MS-SNuPE), MethylLight and methylation-specific PCR (MSP) and they also employ the same principle of modifying DNA with sodium bisulfite prior to PCR amplification [3 – 5, 12, 14, 15]. However, bisulfite DNA sequencing and MSP remains the most common used today in validating microarray data [2]. The use of bisulfite DNA sequencing and MSP methylation analysis techniques in validating the methylation profiles of CpG islands is based on their high sensitivity and specificity, which enable one to analyse every CpG site in a target region. Although the experimental procedure is laborious and time consuming, these techniques also allow for the easy interpretation of data [4, 5].

In a study investigating methylation of CpG islands in the promoter regions of the B-cell gene in Hodgkin lymphoma cell lines, bisulfite DNA sequencing was shown to be able to detect all methylated CpG sites [6]. The use of bisulfite DNA sequencing in validating microarray data has shown high levels of sensitivity [6 - 8].

4.1 AIMS

In this study, bisulfite DNA sequencing was used to:

1. Validate the microarray CpG island methylation data findings using several selected genes in patients with chronic hepatitis B virus (HBV) infection.
2. Validate the microarray CpG data findings using the highly methylated *Cyclin D3* gene in patients with chronic HBV infection and normal controls.
3. Investigate the influence of HBV e antigen positivity on the degree of methylation of promoter region CpG islands of the *Cyclin D3* gene.

4.2 METHODS

4.2.1 Sample Materials

Genomic DNA extracted from the blood samples of HBV patients was used for bisulfite DNA sequencing analysis. Twenty-nine HBV samples were used to validate the presence of CpG island methylation in genes selected from the microarray data discussed in **Chapter 3**. For investigating the influence of HBV e antigen, 10 HBV e antigen positive, 10 HBV e antigen negative and 10 normal control samples were used.

4.2.2 Isolation of DNA from Blood Samples

Genomic DNA was extracted from blood using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA, **Appendix 16**) according to the manufacturer's protocol (see **Appendix 17**). The quality of the DNA samples was assessed using the Nanodrop ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, USA).

4.2.3 Designing of Bisulfite DNA Sequencing Primers

The genes were randomly chosen to validate the microarray methylation data were *cyclin D3* (*CCND3*), *exosome component 3 (EXOSC3), fibroblast growth factor receptor-like-1 (FGFRL1), GA binding protein transcription factor beta subunit 2* (*GABPB2*), *NACC family member 2, BEN* and *BTP* (*POZ*) *domain containing* (*NACC2*), *proliferation associated 2G4* (*PA2G4*), *TBC1 domain family member 17* (*TBC1D17*), *thyroid hormone receptor associated protein 3* (*THRAP3*), *tubulin alpha 4a* (*TUBA4A*), *transient receptor potential cation channel subfamily v member 5* (*TRPV5*) and *UBX domain protein 1* (*UBXN1*). These genes were amongst those that showed higher methylation rates in chronic HBV infected patients when compared to normal, autoimmune hepatitis (AIH) or hepatocellular carcinoma (HCC) cases on the microarray data analysis (see **Chapter 3, Tables 3.3-3.10**).

The primer design algorithm used is outlined in **Figure 4.1**. Using the transcript **start** and **stop** information obtained from the microarray data analysis using Partek Genomic Suite, the sequence of the targeted gene promoter regions were downloaded from Ensembl hg19 assembly [35]. The primers used to target the regions were designed using Applied Biosystem Methyl Primer Express Software® v1.0 [36, 37]. This software and CpG Island Searcher® [38] calculates the density of CpG dinucleotides and determines the presence of CpG islands. If the targeted region has no CpG island, the programme can design primers that will amplify the entire region of interest.

For optimal primer function **(**see **Figure 4.1)**, a number of parameters were taken into consideration when designing the primers. These were that the length of the primers is between 18 and 27 bp to ensure specificity, primers have an annealing temperature of between 56 and 64 °C and that the length of the PCR products was kept short to less than 450bp to ensure maximum yield.

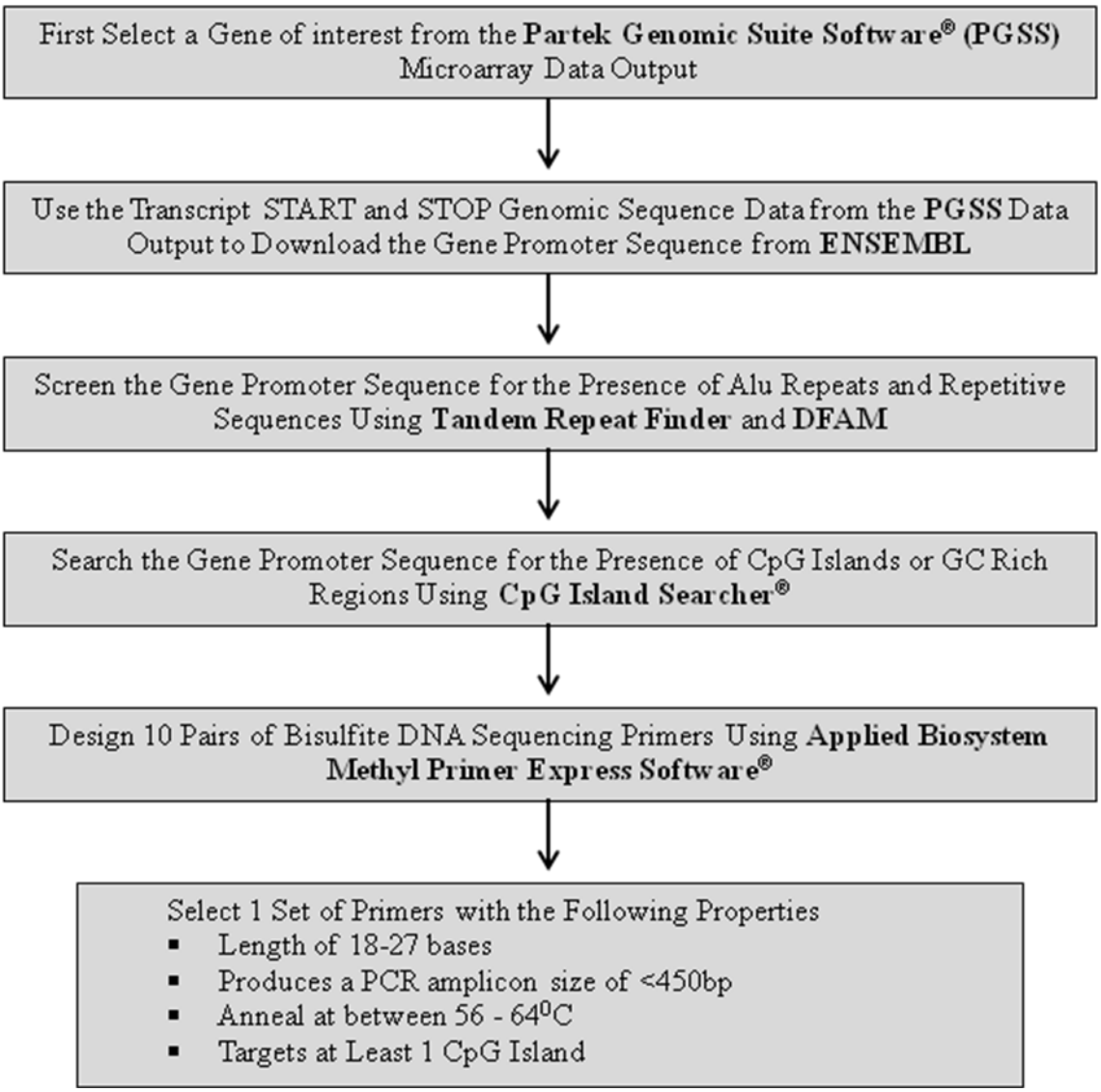

Figure 4.1: Primer Design Algorithm.

Gene promoter sequences were downloaded from Ensembl hg 19 assembly [35]. Tandem Repeat Finder[39] and DFAM[40, 41] were used to screen for the presence of Alu repeats in the targeted region of interest. Applied Biosystem Methyl Primer Express Software® v1.0 [36, 37] was used to design the primers.

The Tandem Repeat Finder® [39] and DFAM® software [40, 41] were used to determine the presence of repetitive DNA sequences or Alu repeats in the sequence of the targeted promoter region and ensure that the primers were not designed within such region. The characteristics of the primers used for the bisulfite DNA sequencing work are outlined in **Table 4.1**.

Table 4.1: Promoter Region Primers used for Bisulfite DNA Sequencing Work

Name of Gene	Primer Sequences	Annealing Temperature (°C)	CpG Percentage	PCR Product Size	Position of CpG Islands
CCND3	Forward: 5'-TTTGAGATGGAGTTTTTTTTTG-3' Reverse: 5'-AATCCCAACACTTTAAAAAACC-3'	60	33	260	42016711- 42017283
EXOSC3	Forward: 5' GGGATTTTTGGAAGTTGAGTAG 3' Reverse: 5' AAAAAAATCCCATAAACCCAC 3'	60	36	381	37785152 - 37785873
FGFRL1	Forward: 5' GTGTTTYGAGAGTTGAGGATT 3' Reverse: 5' TCCCTACRAAACTTTATCAACTC 3'	60	68	398	1005016 - 1005705
GABPB2	Forward: 5' TGTTAGGAGATYGAGATTATTTTA 3' Reverse: 5' CCTAATAAACACATTTTTCCAAT 3'	57	33	335	151042422 -151043032
NACC2	Forward: 5' GGAGGGTTTTTAGTGGGG 3' Reverse: 5' AACTCTCCCRAACTTAACRAAT 3'	61	57	256	138987157 - 138987769
PA2G4	Forward: 5' TGTTTTTGAATTTATTATTTTAGAAAA 3' Reverse: 5' TCCTCCRAAACTAAAAAAACA 3'	57	21	288	56497246 - 56498050
TBC1D17	Forward: 5' TAGTATTTTTTGTTTYGGTTTG 3' Reverse: 5' AAAACCAAAAAAATACCATTTT 3'	57	31	303	50380045 - 50380662
THRAP3	Forward: 5' TTTTAAAATGGTGGGGGTAG 3' Reverse: 5' TAAACCCRCTACCTCCTAAA 3'	60	42	257	220118699 - 220119322
TUBA4A	Forward: 5' AGATGTAGGGTGTTGTGGTAG 3' Reverse: 5' CTATACAATTCTAAATCCTCRACC 3'	58	42	295	142630888 - 142631689
TRPV5	Forward: 5' TTTTAATTTGTGAAATTGGGGT 3' Reverse: 5' TCATTCCTAAAAAAAATCCCAA 3'	61	29	421	142630888 - 142631689
UBXN1	Forward: 5' TGGGAATAGGGAGGTAATTTAT 3' Reverse: 5' CAAAAATCCCTTTAACAATCAA 3'	59	30	281	62246556 - 62247149

Abbreviations: **CCND3** – Cyclin D3, **EXOSC3** – Exosome component 3, **FRFRL1** – Fibroblast growth factor receptor-like 1, **GABPB2** – GA binding protein transcription factor beta subunit 2, **NACC2** - NACC family member 2, BEN and BTP (POZ) domain containing, **PA2G4** – Proliferation associated 2 G4, **PCR** – Polymerase chain reaction, **TBC1D17** – TBC1 domain family member 17, **THRAP3** – Thyroid receptor associated protein 3, **TUBA4A** –Tubulin alpha 4A, **TRPV5** – Transient receptor potential cation channel subfamily v member 5, **UBXN1** – UBX domain protein 1.

4.2.4 Bisulfite Conversion of Genomic DNA

Bisulfite conversion was done using the methods developed by Frommer et al[1], Clarke et al[2], and recently modified by Zhang et al[42]. The recent modification includes a digestion step prior to DNA conversion which reduces the DNA into smaller fragments and facilitates strand separation. *BamHI* restriction endonuclease (details in **Appendix 16**) was used to digest 2µg of genomic DNA by cutting G'GATT'C sites outside the region targeted by the primers. Following this, the digested DNA was subjected to sodium bisulfite treatment using EZ DNA Methylation Gold kit® (Zymo Research, Netherlands, **Appendix 16**) following the manufacturer's instructions. Briefly, 20µl of digested DNA was mixed with bisulfite mix and incubated at 80°C for 10 minutes and 53°C for up to 16 hours in a thermal cycler to complete bisulfite DNA conversion. In this reaction, sodium bisulfite converts unmethylated cytosine residues to uracils in single stranded DNA and leaves methylated cytosine residues unaffected (see **Figure 4.2**). The protocols for

restriction digestion and bisulfite conversion of DNA are described in **Appendices 18** and **19**.

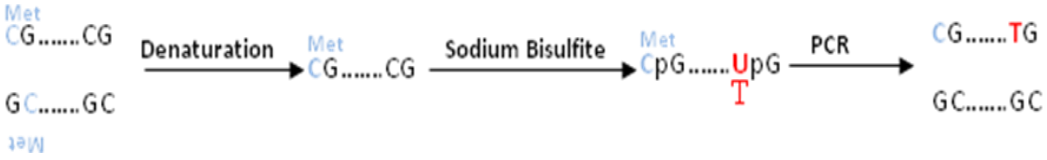

Figure 4.2: Bisulfite Conversion of Genomic DNA (Adapted from Clarke et al[2]).

After denaturation, the DNA is treated with sodium bisulfite which leaves methylated cytosines unaffected and converts unmethylated ones into uracils that appear as thymines in the DNA sequence.

Abbreviations: **C** - Cytosine, **Met** - Methylated, **PCR** - Polymerase chain reaction, **T** - Thymine, **U** - Uracil.

After bisulfite conversion, 1µl of converted products was run on 1.2% agarose gel. After gel electrophoresis, the gel was chilled at 4°C for 15 minutes before viewing under ultraviolet light. Using the primers listed in **Table 4.1**, bisulfite converted DNA was used for PCR amplification (see **Appendix 20**). Longer denaturation steps were incorporated in the PCR conditions to prevent the formation of secondary structures that usually form during bisulfite DNA conversion[42]. The PCR components included HotStar Taq polymerase® (Qiagen, USA) to prevent polymerization at room temperature. After PCR amplification, the products were checked on 1.2% agarose gel and purified using the QIAquick Gel Extraction Kit® (Qiagen, USA, **Appendix 16**) following the manufacturer's instructions (see **Appendix 21**). This purification step removes primer-dimers, deoxynucleotide triphosphates (dNTPs), Taq polymerase and salts from PCR reactions that would otherwise interfere with the subsequent cloning process.

4.2.5 Cloning of Amplified Bisulfite Converted DNA into the Vector

The purified PCR products were cloned into a pGEM®-T Easy Vector using the pGEM®-T Easy Vector System II (Promega, USA, see **Figure 4.3**). The pGEM®-T Easy Vector has a single 3'-terminal thymidine that overhangs at both ends at the cloning site, ensuring highly efficient insertions of PCR products.

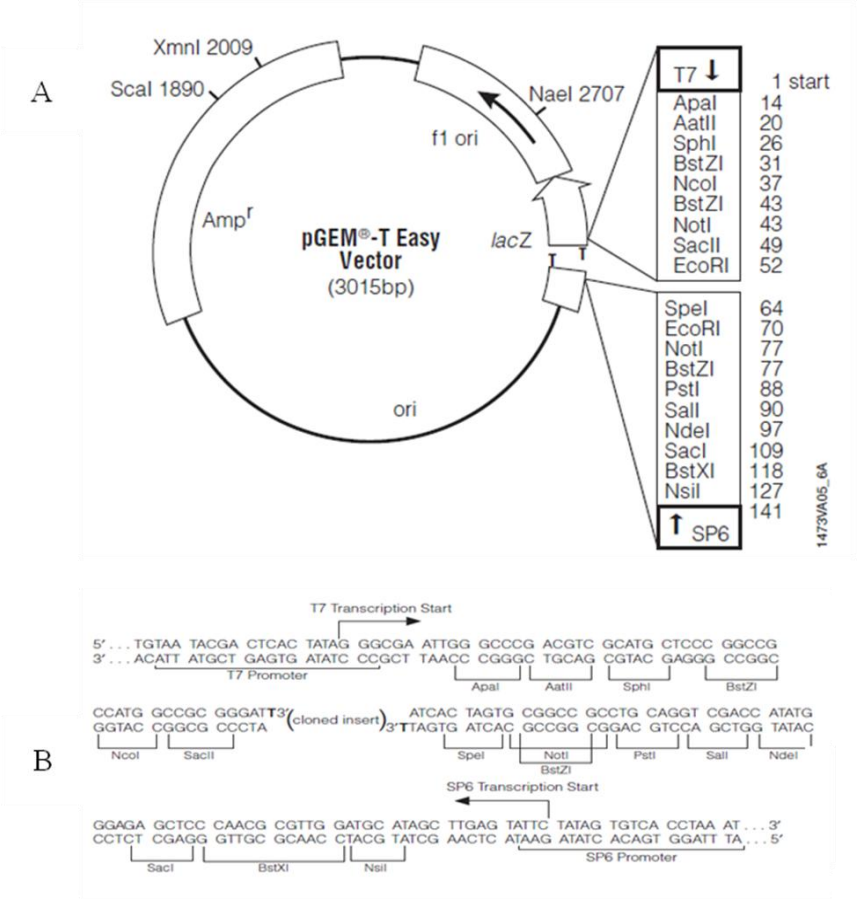

Figure 4.3: Sequence Map and Multi-Cloning Site of pGEM®-T Easy Vector Map.

A pGEM®-T Easy Vector is a linearised vector of 3015bp derived from pGEM®-5ZF (+) vector (**GenBank Accession Number X65308**). It encodes phage T7 and SP6 promoter RNA polymerases at the multiple-cloning sites within the *lacZ* gene encoding β-galactosidase. These promoter RNA polymerases synthesise RNA from the inserted DNA in the 5' to 3' direction and are important in blue/white recombinant selection.

The pGEM®-T Easy Vector also has the *ampR* gene that confers resistance to ampicillin during bacterial growth. It also encodes phage T7 and SP6 promoter RNA polymerases at the multiple-cloning sites within the *lacZ* gene encoding *β*-galactosidase that catalyse the hydrolysis of the lactose analog X-gal.

The promoter RNA polymerases synthesise RNA from the inserted DNA in the 5' to 3' direction and are important in blue/white recombinant selection. X-gal is an inert chromogenic substrate for β-gal. It is colorless and turns blue in the presence of β-gal and is therefore used as a visual marker for non-recombinant colonies. When an insert is cloned into the vector, β-galactosidase is destroyed and is unable to cleave X-gal

hence the clone appears white. White colonies indicate the presence of PCR inserts and blue colonies represent clones with no PCR inserts.

The ligation was carried out using the T4 DNA ligation system (Promega, USA, **Appendix 16**) according to the manufacturer's protocol (see **Appendix 22)**. Ligation reactions were prepared using a 3:1 molar ratio of the insert to the vector. After an overnight incubation for high efficiency ligation, the reactions were transformed into JM109 competent cells (Promega, USA, see **Appendix 23**). Cells transformed with ligation reactions were plated on 2xYT agar plates supplemented with 100µl/ml ampicillin (Sigma Aldrich, USA), 200µg/ml isopropyl-β-D-1-thiogalactopyranoside (IPTG, Sigma Aldrich) and 100µg/ml X-gal (Sigma Aldrich, USA).

4.2.6 Screening of Positive Clones with Vector Plus Insert

Promoter primers which include the pUC/M13, SP6 and T7 enclosing the cloned inserts were used in colony PCR to identify positive clones with vector and inserts. The multiple cloning region of the pGEM®-T Easy vector is flanked by a number of restriction sites for restriction endonucleases that can be used to release the inserts in a single digestion with *NotI*, *BstZI* and *EcoRI* enzymes.

Positive selection of clones with vector plus inserts was carried out using the blue or white color screening. At least and up to 15 white clones were picked and suspended in 20µl deionised water and then 5µl of the recovered supernatant was used as a template in a PCR reaction set (see **Appendix 24)**. A portion of the PCR reaction was run on 1.5% agarose gel. PCR products from these positive clones were purified (see **Appendix 21)** and sent for sequencing.

4.2.7 Sequencing of Positive Clones with Vector Plus Insert

The purified PCR product of 5ng/µl was sent for sequencing using Dye Terminator DNA Sequencer at the Central Sequencing Facility at the University of Stellenbosch, South Africa. The sequencing was performed with 1pmol/µl of both M13 forward (-20) 5'- GTA AAA CGA CGG CCA GT-3' and M13 reverse (-27) 5'- CAG GAA ACA GCT ATG AC-3' primers (Whitehead Scientific, SA, see **Appendix 25.A)**.

DNA sequencing was done using the BigDye Terminator V3.1 sequencing kit® (Applied Biosystem) following manufacture's protocol with slight modifications.

4.3 STATISTICAL METHODS AND DATA ANALYSIS

The sequencing results were extracted in FASTA format using a trace file viewer like Chromas [43]. BioEdit Sequence Alignment Editor was used to create ClustalW multiple alignment of the gene promoter region sequence downloaded from Ensembl hg 19 assembly [35] and the bisulfite converted sequence of positive clones.

The demographic and clinical data of the study patients was analysed using STATA 13 (College Station, Texas, USA, see **Appendix 25.C**). Categorical binary data were summarised as counts and percentages, and categorical ordinal data as median and inter-quantile values. Ordinal numerical data was summarised as median and inter-quantile values and continuous numerical data as means and standard deviations.

The investigation of possible associations between independent categorical variables was performed using the Pearson Chi-Squared test and Fisher's exact test where appropriate. A two-sided P-value of < 0.05 was considered significant.

4.4 RESULTS

4.4.1 Visualising Successful Bisulfite DNA Conversion

Figure 4.4 shows an agarose gel analysis of bisulfite converted DNA products. Initially, only the DNA ladder bands are observed and with no DNA products visible. After chilling the gel on ice, the successfully bisulfite converted DNA products appear as a smear between 100 and 1500 bp **(**see **Figure 4.4B**, **Lanes 2 - 12**). All samples were successfully converted.

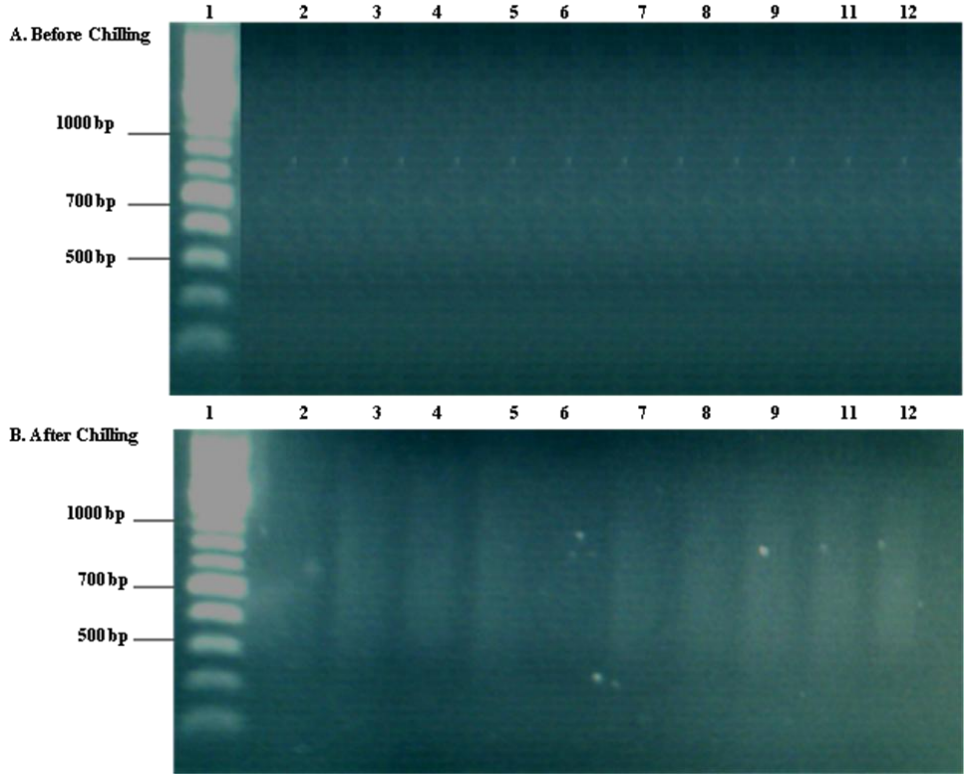

Figure 4.4: Agarose Gel Analysis of DNA Products after Bisulfite Treatment.

2µg of DNA was treated with sodium bisulfite, converted using EZ DNA Methylation Kit and then 2µl from eluted bisulfite converted DNA was run on 1.5% agarose gel containing ethidium bromide. A gel picture was taken immediately after electrophoresis (A, before chilling) and another after chilling at 4°C for 10 minutes (B, after chilling).

Lane 1: 100bp DNA ladder, **Lane 2:** BC_ 522, **Lane 3**: BC_ 496, **Lane 4**: BC_ 505, **Lane 5**: BC_ 496, **Lane 6**: BC_ 428, **Lane 7**: BC_ 428, **Lane 8**: BC_ 499, **Lane** 9: BC_ 444, **Lane 10**: BC_ 594, **Lane 11**: BC_ 505, **Lane 12**: BC_ 493

Abbreviations: **bp** - Base pairs, **BC** - Bisulfite converted, **DNA** - Deoxyribonucleic acid.

4.4.2 Analysis of PCR Amplified Bisulfite Converted Genomic DNA

Agarose gel analysis to confirm successful PCR amplification of bisulfite converted DNA products is shown in **Figure 4.5**. The PCR products produced expected amplicons ranging from 256 - 500 bp depending on the gene promoter primer used is listed in **Table 4.1** (see **Figure 4.5**, **Lanes 2 – 12**). Products in **Lane 4 (***CCND3***)** and **7** (TUBA4A) appeared as more than one band including the expected correct bands of 260 and 295 bp respectively (see **Figure 4.5**, **Lane 4** and **7**)

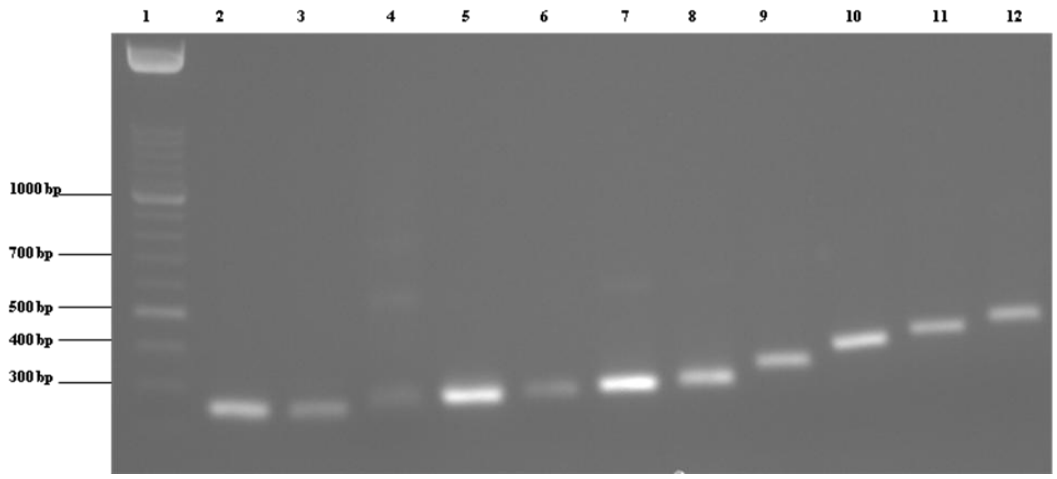

Figure 4.5: Gel Analysis of PCR Amplified Bisulfite Converted DNA.

300ng of bisulfite converted double stranded DNA was amplified using primers specific to eleven different human promoter gene region CpG islands. About 2µl of the PCR products was run on 1.5% agarose gel.

Lane 1: 100bp DNA ladder, **Lane 2:** BC_NACC2_522, **Lane 3**: BC_THRAP3_496, **Lane 4**: BC_CCND3_505, **Lane 5**: BC_UBXN1_496, **Lane 6**: BC_PA2G4_428, **Lane 7**: BC_TUBA4A_428, **Lane 8**: BC_TBC1D17_499, **Lane** 9: BC_GABPB2_444, **Lane 10**: BC_EXOSC3_594, **Lane 11**: BC_FGFRL1_505, **Lane 12**: BC_TRPV5_493.

Abbreviations: **bp** - Base pairs, **BC** - Bisulfite converted, **DNA** - Deoxyribonucleic acid, **CCND3** - Cyclin D3, **EXOSC3** - Exosome component 3, **FGFRL1** - Fibroblast growth factor receptor-like 1, **GABPB2** - GA binding protein transcription factor beta subunit 2, **NACC2** - NACC family member 2 gene promoter, **PA2G4** - Proliferation associated 2G4, **THRAP3** - Thyroid hormone receptor associated protein 1, **TBC1DF17** - TBC 1 domain family member 17, **TRPV5** - Transient receptor potential cation channel subfamily v member 5, **TUBA4A** - Tubulin 4a, **UBXN1** - UBX domain protein 1.

4.4.3 Screening of Positive Clones with Vector Plus Insert by Colony PCR

Subcloning of the PCR products obtained after amplification of bisulfite converted DNA into pGEM T-Easy vector for methylation profile analysis was successful. **Figure 4.6** shows the PCR products amplified from positive clones with correct inserts and these produced expected amplicons ranging from 400 – 600 bp depending on the gene promoter primer product size (see **Table 4.1**), thus confirming successful cloning of the insert into the vector.

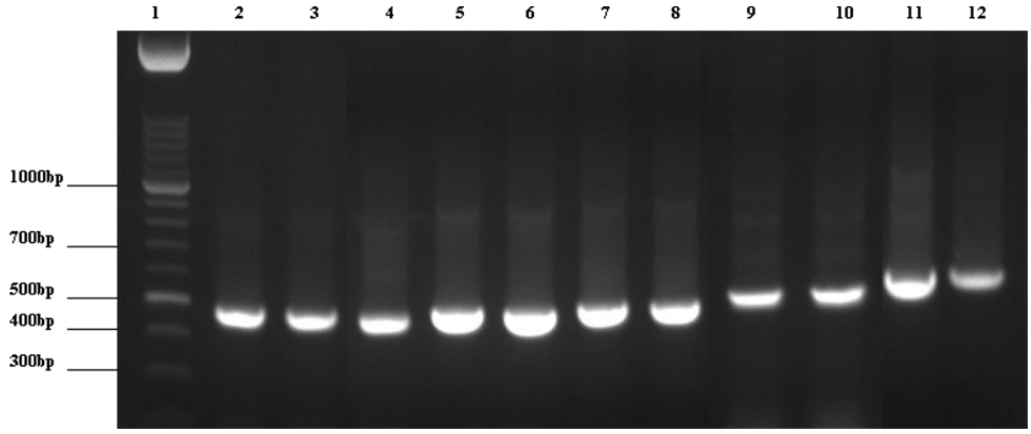

Figure 4.6: Agarose Gel Analysis of Cloned Bisulfite Converted PCR Products into pGEM® T-Easy Vector.

White colonies were picked and resuspended in 20µl of deionised water and centrifuged briefly to obtain a supernatant. 5µl of the supernatant was used as a template in a 50µl PCR reaction following which PCR product was run on 1.5% agarose gel.

Lane 1: 100bp DNA ladder, **Lane 2:** CLN_NACC2_522, **Lane 3**: CLN_THRAP3_496, **Lane 4**: CLN_CCND3_505, **Lane 5**: CLN_UBXN1_496, **Lane 6**: CLN_PA2G4_428, **Lane 7**: CLN_TUBA4A_428, **Lane 8**: CLN_TBC1D17_499, **Lane** 9: CLN_GABPB2_444, **Lane 10**: CLN_EXOSC3_594, **Lane 11**: CLN_FGFRL1_505, **Lane 12**: CLN_TRPV5_493.

Abbreviations: **bp** - Base pairs, **CLN** – Colony, **DNA** - Deoxyribonucleic acid, **CCND3** - Cyclin D3, **EXOSC3** - Exosome component 3, **FGFRL1** - Fibroblast growth factor receptor-like 1, **GABPB2** - GA binding protein transcription factor beta subunit 2, **NACC2** - NACC family member 2 gene promoter, **PA2G4** - Proliferation associated 2G4, **THRAP3** - Thyroid hormone receptor associated protein 1, **TBC1DF17** - TBC 1 domain family member 17, **TRPV5** - Transient receptor potential cation channel subfamily v member 5, **TUBA4A** - Tubulin 4A, **UBXN1** - UBX domain protein 1.

4.4.4 Methylation Pattern of the Gene Promoter CpG Island Regions of Selected Candidate Genes

The methylation profile data of the genes used for validation are summarised in **Table 4.2**. The selected sequence of the *CCND3* promoter CpG islands shows that all of the cytosines detected across the selected sequence were methylated (see **Table 4.2**). In contrast, none of the cytosines in the promoter regions of *GABPB2* and *PA2G4* genes were methylated and only 1(0.2%) was methylated in *EXOSC3* gene (see **Table 4.2**).

Table 4.2: The DNA Methylation Profiles of the Gene Promoter CpG Sequences used for Validation

Gene	Cloning	Sequence Alignment	Methylated Cytosine (N, %)	Bisulfite Converted (N, %)	Age (years)	eAg	Viral DNA Log_{10}	ALT
CCND3	Successful	Yes	19 (100)	0	26	+	8.2	271
EXOSC3	Successful	Yes	1(0.2)	6 (98.9)	18	+	4.1	33
FGFRL1	Successful	Yes	2 (3.1)	65 (96.9)	26	+	8.2	271
GABPB2	Successful	Yes	0	1 (100)	22	+	8.3	25
NACC2	Successful	Yes	2 (5.6)	36 (94.4)	48	+	8.2	118
PA2G4	Successful	Yes	0	14 (100)	33	-	2.1	12
TBC1D17	Unsuccessful	No	-	-	33	-	1.3	22
THRAP3	Successful	Yes	1 (4.8)	21 (95.2)	36	-	3.0	21
TUBA4A	Successful	Yes	2 (18)	11 (82)	33	-	2.6	12
TRPV5	Unsuccessful	No	-	-	17	-	1.8	23
UBXN1	Unsuccessful	No	-	-	36	-	3.4	21

Figure 4.7 shows the DNA methylation profile of a selected section of the bisulfite-sequenced clone of the *CCND3* gene promoter CpG island region. The ClustalW multiple alignment and chromatograph shows no conversion of any cytosine to thymine, indicating that the sequence of the *CCND3* gene promoter CpG island was completely methylated.

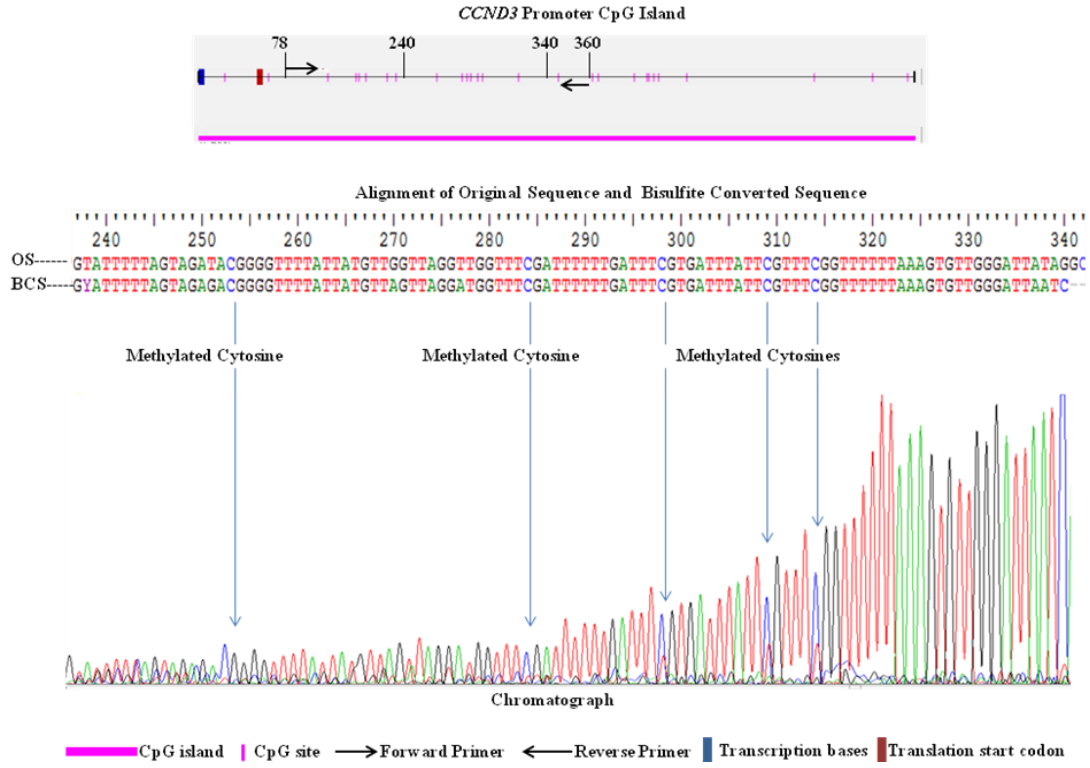

Figure 4.7: Methylation Profile of a Selected Section of *CCND3* Promoter CpG Island Using Bisulfite DNA Sequencing.

Clones with vector plus insert were PCR amplified and sequenced with M13 reverse primer. Methylation profile analysis was performed using trace file viewer like Chromas and sequence alignment of original and bisulfite converted sequences was done with ClustalW multiple alignment in BioEdit Sequence Alignment Editor. After bisulfite conversion, methylated cytosines remain unaffected and unmethylated cytosines are converted to uracils that appear as thymines in the DNA sequence.

Abbreviations: **BCS -** Bisulfite converted sequence, **CCND3** - Cyclin D3, **CpG** - Cytosine-phosphate-Guanine, **DNA** - Deoxyribonucleic acid, **OS** - Original sequence from Ensembl.

A selected section of the sequence alignment for the *FGFRL1* promoter CpG island is shown in **Figure 4.8**. The ClustalW multiple alignment of the Ensembl derived original sequence and bisulfite converted sequences starts from base 107 and end in base 505 of the *FGFRL1* promoter CpG island. Only 2 out of the 67 CpG sites across the *FGFRL1* promoter CpG island were methylated. All the other unmethylated cytosines are converted to thymines indicating complete bisulfite conversion.

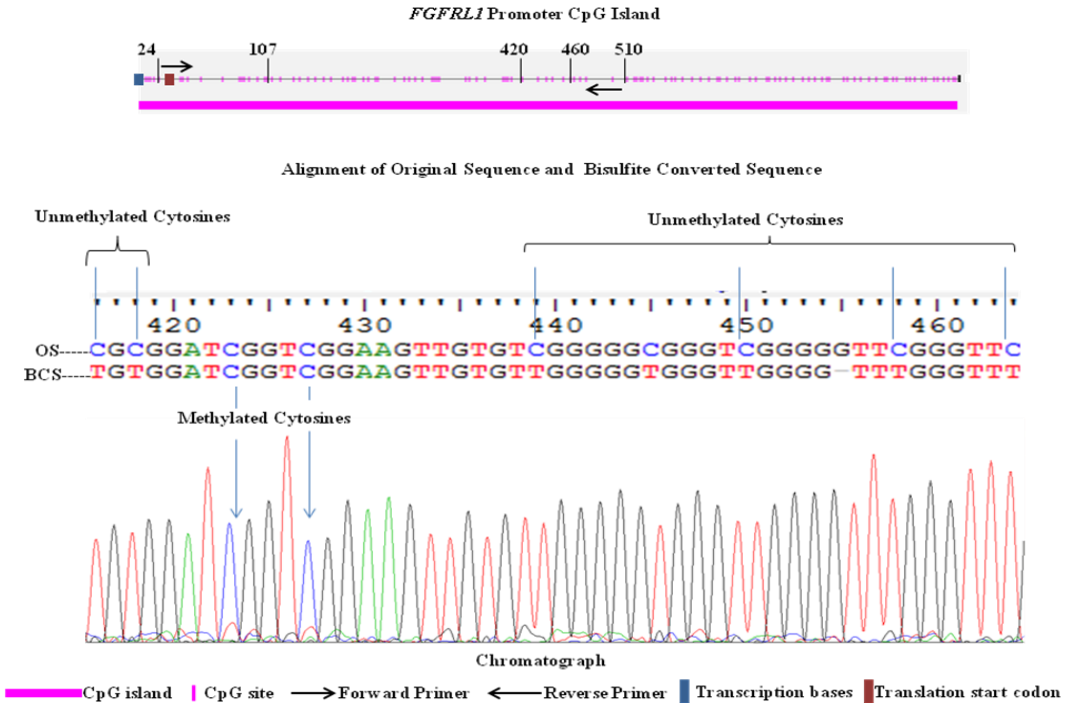

Figure 4.8: Methylation Profiles of a Selected Section of *FGFRL1* Promoter CpG Island Using Bisulfite DNA Sequencing.

Clones with vector plus insert were PCR amplified and sequenced with M13 reverse primer. Methylation profile analysis was performed using trace file viewer like Chromas and sequence alignment of original and bisulfite converted sequences was done with ClustalW multiple alignment in BioEdit Sequence Alignment Editor. After bisulfite conversion, methylated cytosines remain unaffected and unmethylated cytosines are converted to uracils that appear as thymines in the DNA sequence.

Abbreviations: **BCS -** Bisulfite converted sequence, **FGFRL1** – Fibroblast growth factor receptor like-1, **CpG** - Cytosine-phosphate-Guanine, **DNA** - Deoxyribonucleic acid, **OS** – Original sequence from Ensembl.

The ClustalW multiple alignment of the original and bisulfite converted sequences starts from base 136 and ends in base 392 of the *NACC2* promoter (see **Figure 4.9**). The *NACC2* promoter exhibited 2 methylated cytosines out of 38 cytosines of the promoter CpG sites (see **Figure 4.9)**.

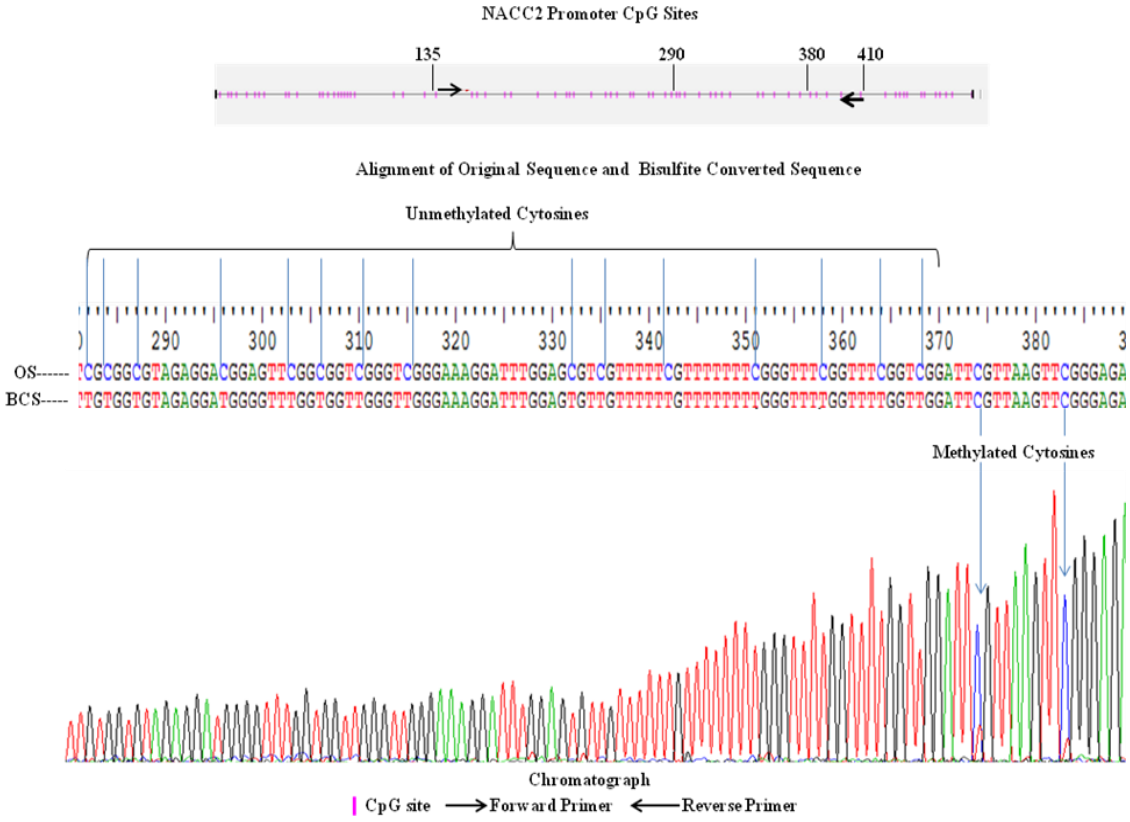

Figure 4.9: Methylation Profile of a Selected Section of *NACC2* Promoter CpG Sites Using Bisulfite DNA Sequencing.

Clones with vector plus insert were PCR amplified and sequenced with M13 reverse primer. Methylation profile analysis was performed using trace file viewer like Chromas and sequence alignment of original and bisulfite converted sequences was done with ClustalW multiple alignment in BioEdit Sequence Alignment Editor. After bisulfite conversion, methylated cytosines remain unaffected and unmethylated cytosines are converted to uracils that appear as thymines in the DNA sequence.

Abbreviations: **BCS -** Bisulfite converted sequence, **NACC2** – NACC family member 2 BEN and BTP (POZ) domain containing, **CpG** - Cytosine-phosphate-Guanine, **DNA** - Deoxyribonucleic acid, **OS** – Original sequence from Ensembl.

The ClustalW multiple alignment of the original and bisulfite converted sequences starts from base 160 and ends in base 360 of the *PA2G4* gene promoter (see **Figure 4.10)**. All the cytosines in the selected section of the *PA2G4* promoter region are unmethylated and converted to thymines (see **Figure 4.10**).

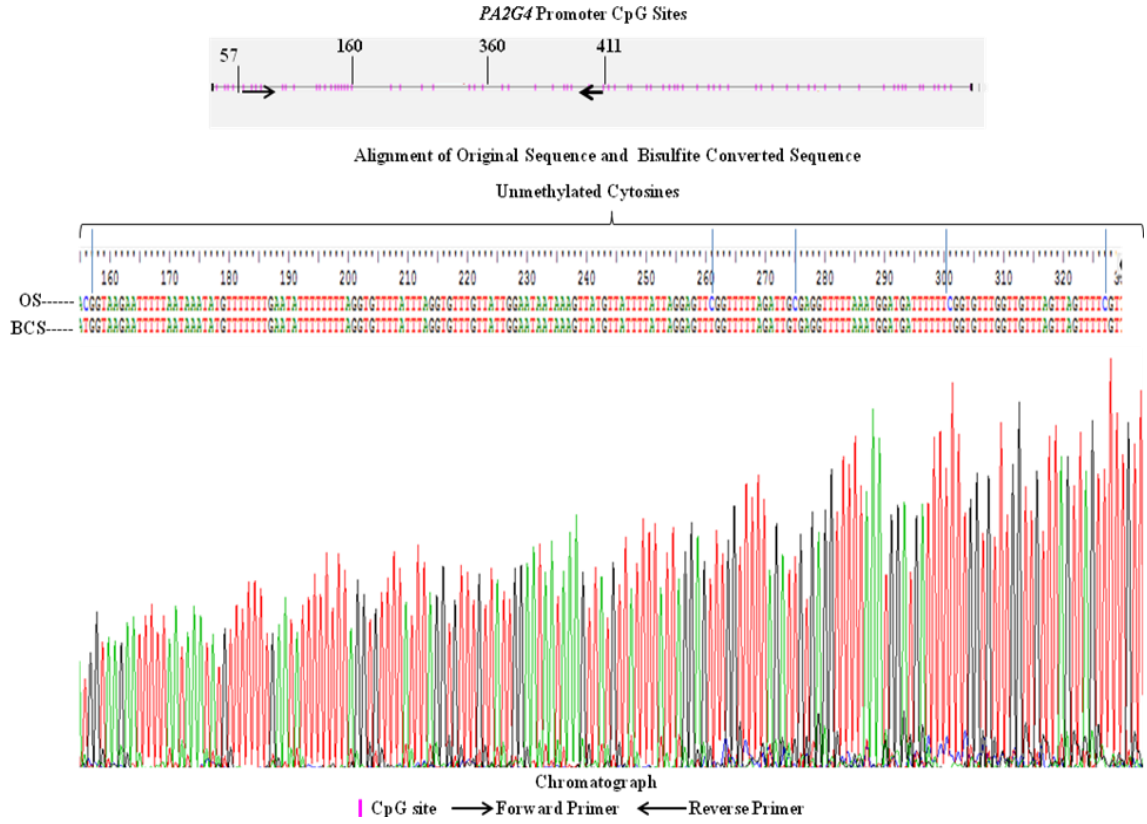

Figure 4.10: Methylation Profile of a Selected Section of *PA2G4* Promoter CpG Sites Using Bisulfite DNA Sequencing.

Clones with vector plus insert were PCR amplified and sequenced with M13 reverse primer. Methylation profile analysis was performed using trace file viewer like Chromas and sequence alignment of original and bisulfite converted sequences was done with ClustalW multiple alignment in BioEdit Sequence Alignment Editor. After bisulfite conversion, methylated cytosines remain unaffected and unmethylated cytosines are converted to uracils that appear as thymines in the DNA sequence.

Abbreviations: **BCS -** Bisulfite converted sequence, **PA2G4** – Proliferation associated 2G4, CpG - Cytosine-phosphate-Guanine, **DNA** - Deoxyribonucleic acid, **OS** - Original sequence from Ensembl.

The ClustalW multiple alignment of the original and bisulfite converted sequences starts from base 111 and ends in base 366 of the *THRAP3* gene promoter CpG island (see **Figure 4.11**). The ClustalW multiple alignment of the original sequence for *THRAP3* gene promoter exhibited methylation of 1 of the 22 cytosines in the selected section of the CpG island in this gene promoter illustrated here (see **Figure 4.11)**.

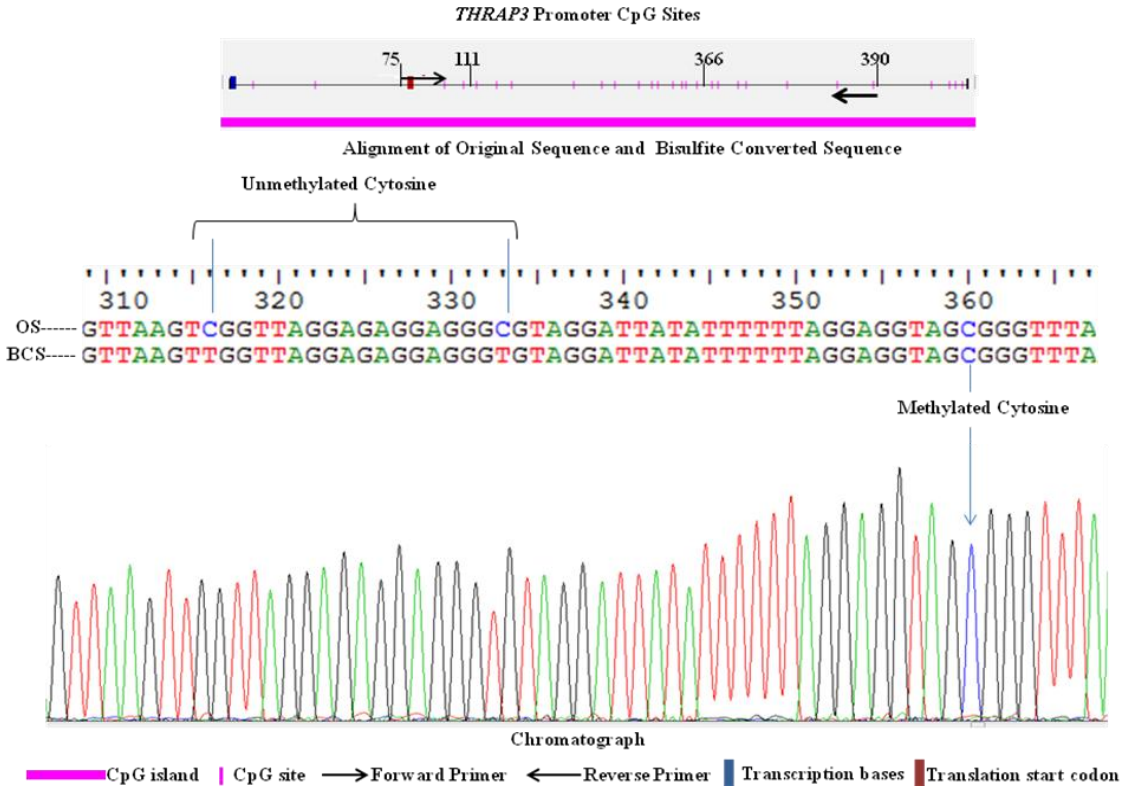

Figure 4.11: Methylation Profile of a Selected Section of *THRAP3* Promoter CpG Island Using Bisulfite DNA Sequencing.

Clones with vector plus insert were PCR amplified and sequenced with M13 reverse primer. Methylation profile analysis was performed using trace file viewer like Chromas and sequence alignment of original and bisulfite converted sequences was done with ClustalW multiple alignment in BioEdit Sequence Alignment Editor. After bisulfite conversion, methylated cytosines remain unaffected and unmethylated cytosines are converted to uracils that appear as thymines in the DNA sequence.

Abbreviations: **BCS -** Bisulfite converted sequence, **THRAP3** – Thyroid hormone receptor associated protein 3, **CpG** - Cytosine-phosphate-Guanine, **DNA** - Deoxyribonucleic acid, **OS** - Original sequence from Ensembl.

The ClustalW multiple alignment of the original and bisulfite converted sequences starts from base 160 and ends in base 300 of the *TUBA4A* gene promoter CpG island (see **Figure 4.12**). There were 2 methylated cytosines in the selected section of the aligned sequence of the clone with *TUBA4A* gene promoter CpG island.

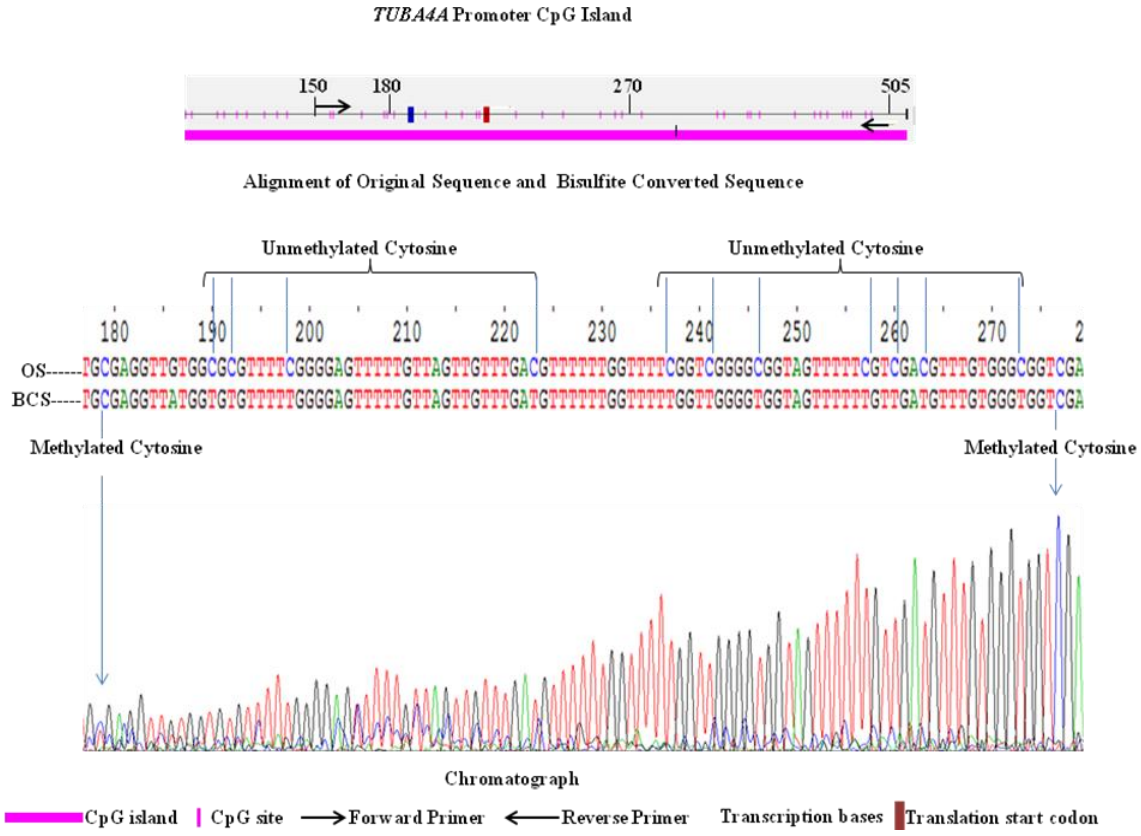

Figure 4.12: Methylation Profile of a Selected Section of *TUBA4A* Promoter CpG Island Using Bisulfite DNA Sequencing.

Clones with vector plus insert were PCR amplified and sequenced with M13 reverse primer. Methylation profile analysis was performed using trace file viewer like Chromas and sequence alignment of original and bisulfite converted sequences done with ClustalW multiple alignment in BioEdit Sequence Alignment Editor. After bisulfite conversion, methylated cytosines remain unaffected and unmethylated cytosines are converted to uracils that appear as thymines in the DNA sequence.

Abbreviations: **BCS -** Bisulfite converted sequence, **TUBA4a** – Tubulin 4a, **CpG** - Cytosine-phosphate-Guanine, **DNA** - Deoxyribonucleic acid, **OS -** Original sequence from Ensembl.

The ClustalW multiple alignment of the Ensembl derived original and bisulfite converted sequences starts from base 267 and ends in base 354 of the *GABPB2* promoter CpG island (see **Figure 4.13**). Only one CpG site was observed on the selected region of sequence alignment and the cytosine on this site was converted to thymine and is therefore not methylated (see **Figure 4.13**).

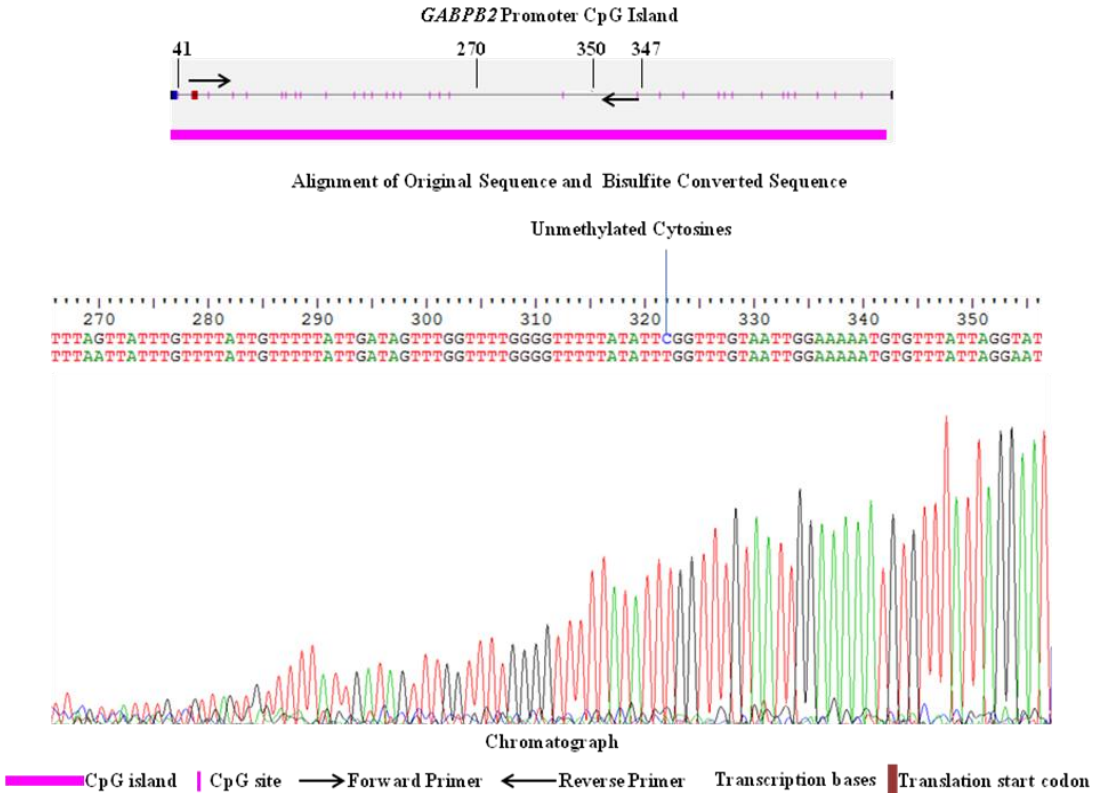

Figure 4.13: Methylation Profile of a Selected Section of *GABPB2* Promoter CpG Island Using Bisulfite DNA Sequencing.

Clones with vector plus insert were PCR amplified and sequenced with M13 reverse primer. Methylation profile analysis was performed using trace file viewer like Chromas and sequence alignment of original and bisulfite converted sequences was done with ClustalW multiple alignment in BioEdit Sequence Alignment Editor. After bisulfite conversion, methylated cytosines remain unaffected and unmethylated cytosines are converted to uracils that appear as thymines in the DNA sequence.

Abbreviations: **BCS -** Bisulfite converted sequence, **GABPB2** – GA binding protein transcription factor beta subunit 2, **CpG** - Cytosine-phosphate-Guanine, **DNA** - Deoxyribonucleic acid, **OS -** Original sequence from Ensembl.

The ClustalW multiple alignment of the original and bisulfite converted sequences starts from base 193 and ends in base 449 of the *EXOSC3* gene promoter CpG island (see **Figure 4.14**). The *EXOSC3* gene promoter CpG island exhibited 7 CpG sites on the selected section of aligned region (see **Figure 4.14**). Only 1 cytosine of the CpG sites was methylated and the rest were converted to thymines.

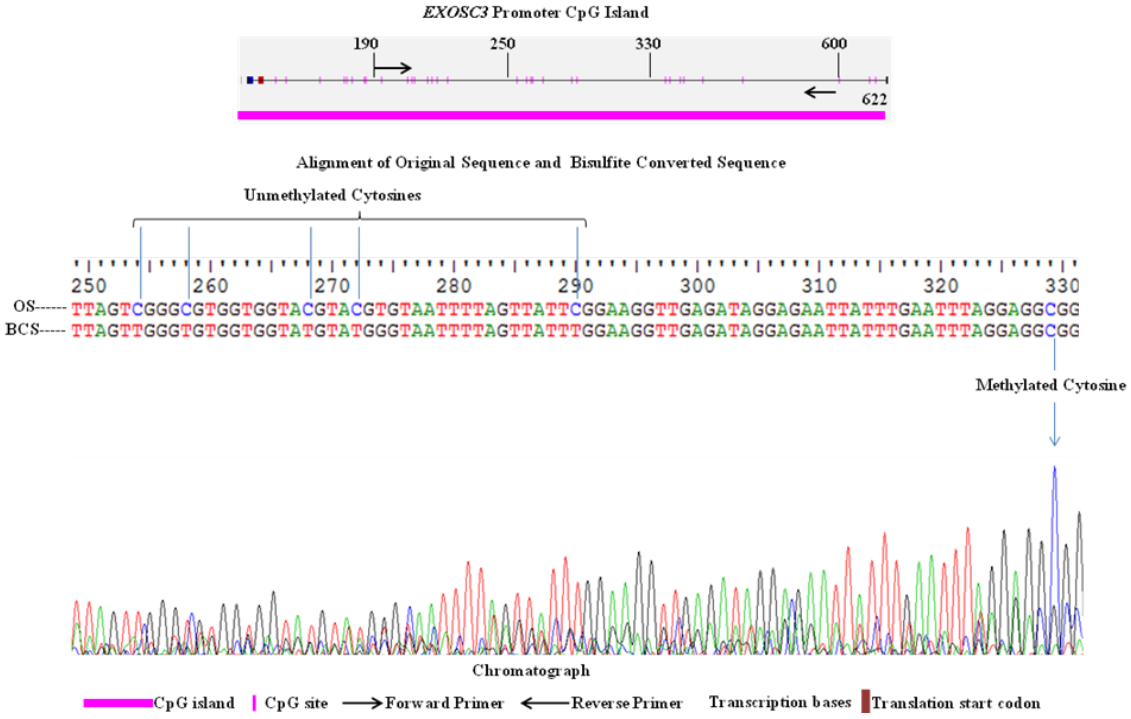

Figure 4.14: Methylation Profile of a Selected Section of *EXOSC3* Promoter CpG Island Using Bisulfite DNA Sequencing.

Clones with vector plus insert were PCR amplified and sequenced with M13 reverse primer. Methylation profile analysis was performed using trace file viewer like Chromas and sequence alignment of original and bisulfite converted sequences was done with ClustalW multiple alignment in BioEdit Sequence Alignment Editor. After bisulfite conversion, methylated cytosines remain unaffected and unmethylated cytosines are converted to uracils that appear as thymines in the DNA sequence.

Abbreviations: **BCS -** Bisulfite converted sequence, **EXOSC3** – Exosome component 3, **CpG** - Cytosine-phosphate-Guanine, **DNA** - Deoxyribonucleic acid, **OS -** Original sequence from Ensembl.

4.4.5 Methylation Profile of *Cyclin D3* in HBV e Antigen Positive and Negative Patients

The profile of the cohort used to investigate the highly methylated *CCND3* gene is outlined in **Table 4.3**. There was equal proportion of HBV e antigen positive, negative and normal controls. Bisulfite DNA sequencing analysis was unsuccessful in 30% HBV e antigen positive, 50% HBV e antigen negative and 70% normal control DNA samples (see **Figure 4.3**).

Table 4.3: Profile of Patient Cohort Used to Investigate *CCND3* Gene

Experimental Data	**Sample Cohort (Number = 30)**	**HBVeAg Positive (Number = 10)**	**HBVeAg Negative (Number = 10)**	**Negative Controls (Number = 10)**
	Number (%)	**Number (%)**	**Number (%)**	**Number (%)**
Bisulfite Conversion and Amplified by PCR	30 (100)	10 (100)	10 (100)	10 (100)
Cloned into the Vector	18 (60)	8 (80)	7 (60)	3 (30)
Successful in Sequencing and Used for Analysis	15 (50)	7 (70)	5 (50)	3 (30)

Abbreviations: **%** - Percentage, **HBVeAg** – Hepatitis B virus e antigen, **PCR** – Polymerase chain reaction

The ClustalW multiple alignment of bisulfite converted sequences of clones with *CCND3* gene promoter CpG islands of normal controls, HBeAg positive and negative patients is shown in **Figure 4.15**. Cytosine methylation is present only in HBV patients and all the cytosines in the normal control sequences were converted to thymines, indicating the absence of methylation at those sites (see **Figure 4.15**).

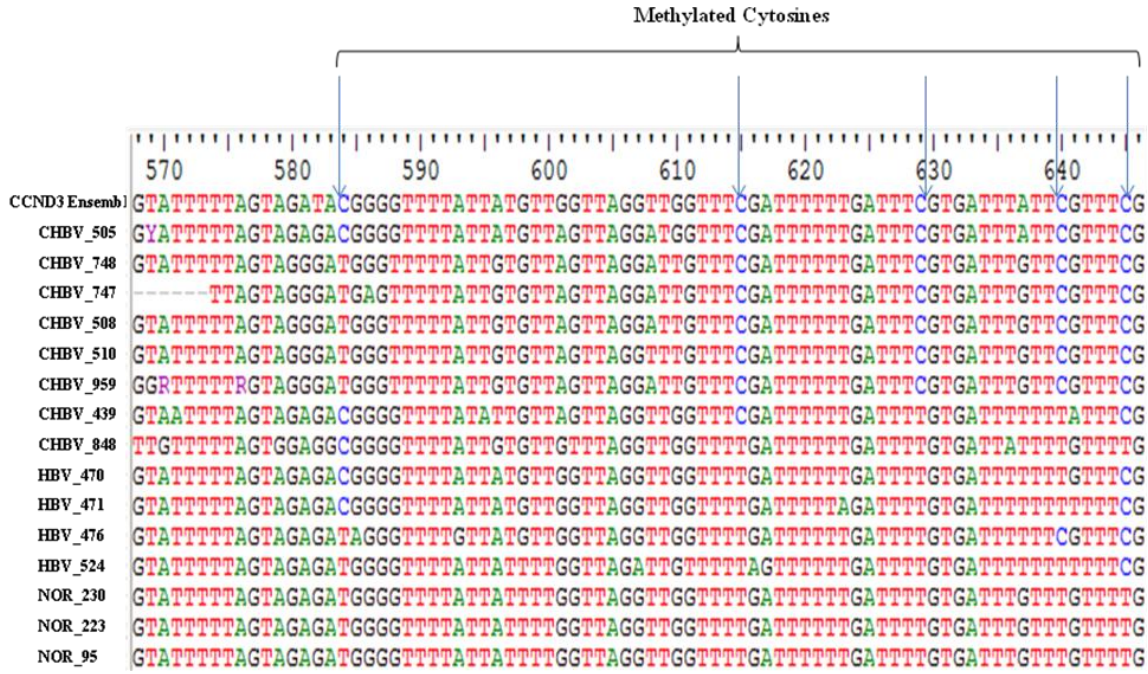

Figure 4.15: Methylation Profile in a Selected Section of the *CCND3* Promoter Region in HBV Patients and Controls.

Single clone with vector plus insert was PCR amplified and sequenced with M13 reverse primer. For each CpG site, CHBV, HBV and Normal sera were compared using ClustalW multiple aligned sequences generated in BioEdit Sequence Alignment Editor.

Abbreviations: **CCND3 Ensembl** - Cyclin D3 sequence derived from Ensembl, **CHBV** – HBV e antigen positive, **CpG** - Cytosine-phosphate-Guanine, **HBV** – HBV e antigen negative.

In the successfully cloned and sequenced samples, most of the patients were male 11 (78.6%) and young with a median age of 22.3 yrs (see **Table 4.4**). There was no gender ($p = 0.5$) or age difference ($p = 0.7$) between the HBV e antigen positive and negative groups. The HBV e antigen positive group had significantly higher HBV DNA levels ($p = 0.02$) and *CCND3* gene promoter methylation ($p = 0.001$) compared to the HBV e antigen negative group. The HBV e antigen positive group had higher ALT levels ($p = 0.09$) and INR levels ($p = 0.09$) but these did not reach significance levels.

Table 4.4: Demographic and Methylation Profile of the *CCND3* Gene Promoter in HBV Infected Cohort

	All N = 13	**HBV eAg Negative N = 6**	**HBV eAg Positive N = 8**	**P Value**
	(**Number, %**)	(**Number, %**)	(**Number, %**)	
Male Gender	11 (78.6)	5 (71.53)	6 (85.71)	0.5
	(**Median, IQR**)	(**Median, IQR**)	(**Median, IQR**)	
Age (years)	22.3 (18.8 - 44.4)	21.1 (18.4 - 44.8)	24.7 (18.8 - 44.4)	0.7
HBV DNA Log_{10}	8.2 (5.2 -8.3)	5.15 (3.9 -16.9)	8.3 (8.2 -8.5)	0.022
Methylation %	50 (40 - 80)	40 (20 - 40)	80 (80 - 80)	0.001
α FP (u/L)	2.5 (2.1 – 3.3)	2.5 (1.3 – 2.8)	3 (2.4 – 4.6)	0.19
Albumin (35 – 50 g/L)	44.5 (42 - 46)	45 (40 - 46)	42 (44 - 49)	0.65
ALT (0 – 40 u/L)	42.5 (27 – 108)	32 (21 – 45)	67 (37 - 271)	0.09
INR (< 1.2)	1.02 (0.9 – 1.1)	0.96 (0.9 – 1.1)	1.1 (1 - 1.35)	0.09
Platelets (150 – 400 x 10^9 L)	239 (212 – 254)	237 (212 – 244)	254 (204 - 400)	0.57

Abbreviations: **%** - Percentage, **αFP** – Alpha Fetoprotein, **ALT** – Alanine aminotransferase, **INR** – International normalised ratio, **IQR** - Inter -quantile range, **HBVeAg** - Hepatitis B virus e antigen

When the cohort was analysed by the different HBV genotypes, there was no difference in the gender profile (p = 0.6), age at diagnosis (p = 0.3), alpha fetoprotein levels (p = 0.65) and ALT levels (p = 0.4) between HBV genotypes A and D infected patients (see **Table 4.5**). Genotype D patients had significantly higher HBV DNA levels (p = 0.006) and *CCND3* gene promoter methylation (p = 0.005) compared to genotype A patients. Genotype D had higher INR levels (p = 0.07) but this did not reach statistical significance.

Table 4.5: Cohorts HBV Genotype and *CCND3* Gene Promoter Methylation Profiles

	Genotype A	**Genotype D**	**P Value**
	(**Number, %**)	(**Number, %**)	
Male Gender	6 (75)	5 (83.3)	0.6
	(**Median, IQR**)	(**Median, IQR**)	
Age (years)	20.3 (18.5 - 33.6)	25.3 (23.3 - 44.4)	0.3
Log_{10} HBV DNA	5.2 (4.6 - 5.2)	8.3 (8.2 -8.5)	0.006
Methylation %	40 (30 - 80)	80 (80 - 80)	0.005
αFP (u/L)	2.5 (1.7 - 3.1)	2.7 (2.4 – 3.5)	0.65
Albumin (35 – 50 g/L)	44.5 (11 - 45.5)	45 (43 - 49)	0.36
ALT (0 – 40 u/L)	36 (24 - 146.5)	60.5 (37 - 108)	0.4
INR (< 1.2)	0.98 (0.92 - 1.05)	1.19 (1.03 - 1.35)	0.07
Platelets (150 – 400 x 10^9 L)	239.5 (219 - 303)	24.4 (204 - 254)	0.79

Abbreviations: **%** - Percentage, αFP – Alpha Fetoprotein, **ALT** – Alanine aminotransferase, **INR** – International normalised ratio, **IQR** - Inter -quantile range, **HBV** - Hepatitis B virus

4.5 DISCUSSION

The main aim of this study was to validate the methylation profiles of promoter CpG islands of selected genes identified in the microarray data analysis. The promoter regions of interest were those of *CCND3*, *EXOSC3*, *FGFRL1*, *PA2G4*, *GABPB2*, *NACC2*, *TBC1D17*, *TUBA4A*, *THRAP3*, *TRPV5* and *UBXN1* genes. Using bisulfite DNA conversion, cloning of amplified converted DNA and sequencing, every cytosine methylated in the target sequence of the selected gene promoter regions was successfully identified (see **Figures 4.7 - 4.15**), confirming the presence of DNA methylation in chronic HBV infected patients. In addition, the highly methylated *CCND3* gene was further investigated in HBV infected patients and normal controls (see **Figure 4.15**)

4.5.1 Visualizing Successful Bisulfite DNA Conversion

The chilling effect method first discovered and described by Ruga et al[10] was used to visualize successful bisulfite conversion of DNA in an agarose gel prior to PCR amplification and cloning into the vector (see **Figure 4.4B**). As bisulfite converted DNA is single stranded, it is impossible to view it in an agarose gel with ethidium bromide because the complementarily of bases is lost and the two strands are too far apart than in normal double stranded DNA that allows intercalation of the ethidium bromide [10].

Dropping the temperature through chilling allows single stranded DNA to fold back onto itself and retain a local base pairing allowing intercalation of the ethidium bromide and therefore visualization under ultraviolet [10]. Knowledge of this effect allows one to visualize successfully converted DNA samples prior to PCR amplification and cloning into the vector.

4.5.2 Validation of Methylation Profiles Obtained Through Microarray Data Analysis

Eleven promoter regions of *CCND3*, *EXOSC3*, *FGFRL1*, *PA2G4*, *GABPB2*, *NACC2*, *TBC1D17*, *TUBA4A*, *THRAP3*, *TRPV5* and *UBXN1* genes were selected to give a

representation of genes in each of the functional groups of cyclin-dependent kinase, growth factors, tumour suppressors, transcriptional factors, vitamin D receptor binding, mRNA splicing and metabolic regulators that were identified in the microarray analysis (see **Chapter 3**, **Tables 3.12**) [16 - 24].

The ClustalW multiple sequence alignment comparing the gene promoter sequences downloaded from Ensembl hg19 assembly [35] and DNA sequences amplified from positive colonies harbouring clones with *CCND3*, *EXOSC3*, *FGFRL1*, *NACC2*, *TUBA4A* and *THRAP3* genes showed the presence of methylation, confirming the methylation data from microarray analysis.

The methylation rate of cytosine bases varied from 0 to 100 % between different genes (see **Table 4.2**). The targeted sequences of *FGFRL1*, *NACC2*, *THRAP3*, *TUBA4A* and *EXOSC3* showed CpG site methylation rates of 3 – 18 % (see **Table 4.2**). Lower rates of methylation in the promoter regions of these genes may be related to the stage of disease progression and function of the genes. Alternatively, different primers targeting other areas of the promoter could have found higher rates of methylation in the same patients. Although some of these genes may be methylated early in the stage of disease, methylation may accumulate as the liver disease progresses and could therefore become higher in the advanced liver disease [47]. This needs further investigation in older patients or cirrhotic patients using different bisulfite sequencing primers.

Higher methylation rates may be influenced by the duration of infection, virus genotype, viral load and environmental factors such as aflatoxins, drugs, chronic smoking and alcohol consumption which can also induce methylation [15, 28 -34]. In this study, the *CCND3* gene promoter region analysed was found to be heavily methylated (see **Table 4.2**). The DNA used for the bisulfite DNA sequence analysis in the validation cohort of *CCND3* gene was obtained from a young patient with a high ALT and HBV viral load (see **Table 4.2**). In the *CCND3* gene validation cohort, methylation was associated with HBV e antigen positive status, viral load and genotype (see **Table 4.4**). These observations are supported by several studies which show that HBV genotype A infected patients, viral loads of greater than 1×10^4 copies per ml and HBV e antigen positive status are associated with increased methylation and a high risk of malignant transformation of hepatocytes [30, 33, 43 - 45].

The type of HBV insert being methylated could be another risk factor for increased methylation in *CCND3* gene validation cohort as it may be possible that HBV x, core or surface antigens insert differently into the genome and therefore stimulate different host immune responses [46]**.** This hypothesis would find support from the recent work of Tseng et al study which shows that the risk of developing HCC is high in patients with raised levels of HBV surface antigen, even in the presence of low HBV DNA levels [58]. This finding could be a reflection of high HBV surface antigen levels causing higher levels of HBV surface integration into the human genome resulting in higher rate of malignant transformation [59 - 61]. Therefore, the higher methylation levels of *CCND3* (see **Table 4.2**) and other genes identified in HBV infected patients compared to normal controls in this study (see **Chapter 3**, **Table 3.3**) may be due to genomic instability caused by HBV inserts into the genome of surface antigen [62].

Although bisulfite DNA conversion for the promoters of *TRPV5*, *UBXN1* and *TBC1D17* genes was successful, there were no positive clones with a correct insert in the vector. Similar data was obtained with some of *CCND3* gene validation cohort (see **Table 4.3**). One possible reason for this failure is that ultraviolet light overexposure of the DNA on the agarose gel prevented successful ligation. On ultraviolet light overexposure, pyrimidine dimmers may form [48]. These can prevent ligation of DNA extracted from ultraviolet exposed gel. This can be avoided by limiting the time of ultraviolet light exposure using a glass plate between the gel and ultraviolet source. Another possibility is that, although, the PCR fragment may be successfully inserted into the vector, it fails to disrupt the *lacZ* gene, resulting in a higher number of blue colonies. This can be caused by low activity of DNA ligase and buffer due to multiple freeze-thaw cycles. This can be addressed by making use-single aliquots of DNA ligase and buffer, and using a fresh aliquot with every ligation [48]. It is also possible that the successful inserts were from DNA fragments that were not the targeted sequence. This can be addressed by ensuring that there are no other undesired bands in the PCR product before ligation [49]

The *PA2G4* and *GABPB2* gene promoter regions exhibited no methylation and all the cytosines in the CpG sites of these regions were converted to thymines, further validating successful bisulfite conversion. The absence of DNA methylation in the

promoter regions of *PA2G4* and *GABPB2* genes may be due to the fact that the selected patient happened not to have methylation in these genes. Another explanation could be due to a possible lower rate of methylation in blood when compared to liver tissue, which as a replication site for HBV may exhibit more methylation [63]. It is also possible that the selected region have no methylated sites and this can be addressed by using different set of primers to target the entire promoter region. Patients may also not have HBV related methylation due to having a short period of infection and therefore not have accumulated much methylation. There could also be patient specific factors that could delay or prevent methylation such as the absence of cigarette smoking or no use of drugs that could facilitate methylation in the presence of HBV infection. Viral related factors would also be important. These could be the viral genotype, level of viral replication and the type of inserts in the genome such as HBV surface antigen compared to HBV x antigen that could affect the innate immune response and hence the activation of methylation (see **Chapter 1**, **Section 1.4.3**). These genes need to be studied further with a larger cohort, patients with different viral loads and genotypes.

There was no methylation observed in the core promoter regions within 100bp upstream of the TSS in chromosomes X and Y (see **Chapter 3**, **Figures 3.9** and **3.10**). A possible explanation for this finding could be that these chromosomes are protected by the normal epigenetic silencing that occurs during embryogenesis as is seen with X chromosome inactivation and may therefore and remain relatively inactive and protected from further methylation throughout the lifetime of the cell [64 - 66] (see **Chapter 1, Section 2.2.2**).

4.5.3 Methylation of *CCND3* Gene Promoter Region Compared by HBV e Antigen Status

In the study cohort, normal cases had no methylation and HBV e antigen positive cases were significantly more methylated. This methylation difference was associated with genotype D and a high viral load (see **Table 4.4**), suggesting that the higher methylation rate of the *CCND3* gene promoter is strongly associated with HBV e antigen positive status**.** The HBV e antigen positive patients were also

significantly associated with higher viral DNA load and genotype D while age at analysis had no influence (see **Table 4.4**).

The high methylation rate of the *CCND3* gene promoter region in this study suggests that the *CCND3* gene could be important in the pathogenesis of liver disease in chronic HBV infection. The *CCND3* gene is a cyclin-dependent kinase gene that is important in liver regeneration and wound healing processes and has been linked to the development of liver fibrosis and HCC [22, 26]. The abnormal function of the *CCND3* gene promoter due to methylation would disrupt normal cellular signalling with effects on cell growth, differentiation, proliferation and apoptosis. Such changes would promote the clonal expansion of mutant hepatocytes, increase hepatic inflammation and fibrosis [26, 27].

The association with HBV e antigen positivity could be a reflection of the high viral DNA load thereby confirming the known HBV viral physiology of high replication in HBV e antigen positive patients [33]. The association of high methylation rates with viral replication is significant in that the risk of cirrhosis and liver cancer is strongly correlated with viral replication [33].

Notably, genotype D cases had significantly higher HBV DNA levels and *CCND3* gene promoter methylation compared to genotype A cases (see **Table 4.5**). This can be explained by clinical differences of HBV genotypes which have been previously reported in respect to their influence in HCC and response to chronic HBV treatment [50 - 53]. Sustained biochemical remission and clearance of HBV DNA or HBV surface antigen was found to be frequent in HBV genotype A than D which is associated with more severe liver disease [50]. Moreover, genotype D was found to be frequent in HCC patients below 40 years of age [51], suggesting that it could be more damaging to the liver. In terms of therapy, the response to interferon was found to be significantly higher in genotype A than D chronic HBV infected patients, and in patients with lower levels of HBV DNA and higher levels of ALT [52 - 58]. Given this evidence, it is interesting to suggest that high DNA methylation rates may have an influence on the ability to respond to anti-HBV treatment. The level of DNA methylation and the HBV genotype could possibly predict the response to antiviral therapy and if so be used to individualise therapy by type and duration.

4.6 CONCLUSION

This study has confirmed the microarray findings that patients with chronic HBV infection have significantly methylated gene promoter CpG islands compared to other patient groups.

In patients with chronic HBV infection, methylation was significantly associated with the HBV viral load, HBV e antigen positive status and genotype D. Some genes were highly methylated and this could relate to their importance in liver disease in the presence of chronic HBV infection. In particular, the *CCND3* gene was highly methylated and its potential disruption could explain the development of hepatic inflammation, fibrosis and malignant transformation.

These mechanisms require further investigation to investigate the link between liver disease and DNA methylation caused by viral or environmental factors such as duration of infection, virus genotype, viral load, insertion of HBV antigens, drugs, aflatoxins, chronic smoking and alcohol consumption.

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CHAPTER 5

DISCUSSION AND CONCLUSIONS

5. INTRODUCTION

Hepatitis B virus (HBV) is one of the most prevalent infections in humans and important cause of acute and chronic hepatitis [1, 2, 126]. Long term inflammatory changes due to chronic hepatitis cause hepatocyte injury and the release of reactive oxygen species and Kupffer cells activation [3, 4]. These produce proinflammatory and fibrogenic cytokines resulting in the recruitment of immune cells [4]. The Kupffer cells also activate hepatic stellate cells which produce extracellular matrix proteins and cytokines [5, 10]. Repeating cycles of this activation and inflammation leads to cirrhosis characterised by regenerative nodules and irreversible fibrosis [6 – 8, 126, 127].

The ability of the virus to cause liver injury is associated with genetic changes that affect both viral and host DNA leading to mutations that predispose to liver injury and possible cancer [9, 10]. These events link chronic HBV infection with hepatocellular carcinoma (HCC). HCC is the sixth most common cancer in the world with highest incidence rates being in Asia and sub-Saharan Africa [11, 12]. More than 80% of HCC cases arise in chronic HBV infection, strongly suggesting that HBV is an important contributor to the development of tumour [11 - 14].

Possible mechanisms by which HBV infection causes HCC have been described and these include HBV DNA integration, epigenetic changes and aberrant transcriptional activities of HBx protein [11, 15, 16, 24, 25] (see **Figure 5.1**). Nearly 90% of HBV-related HCC cases show evidence of HBV integration into the host genome [11]. This is associated with genetic changes such as genomic instability, deletions and chromosomal translocations in the host cells, which may lead to accumulation of mutations and epigenetic changes with a malignant phenotype [16]. Several contributing environmental and viral factors such as chronic tobacco smoking, alcohol consumption, aflatoxins, HBV e antigen positive status, high viral load and HBV genotype have been identified in HBV-related HCC cases and are associated with many epigenetic changes [17 - 23].

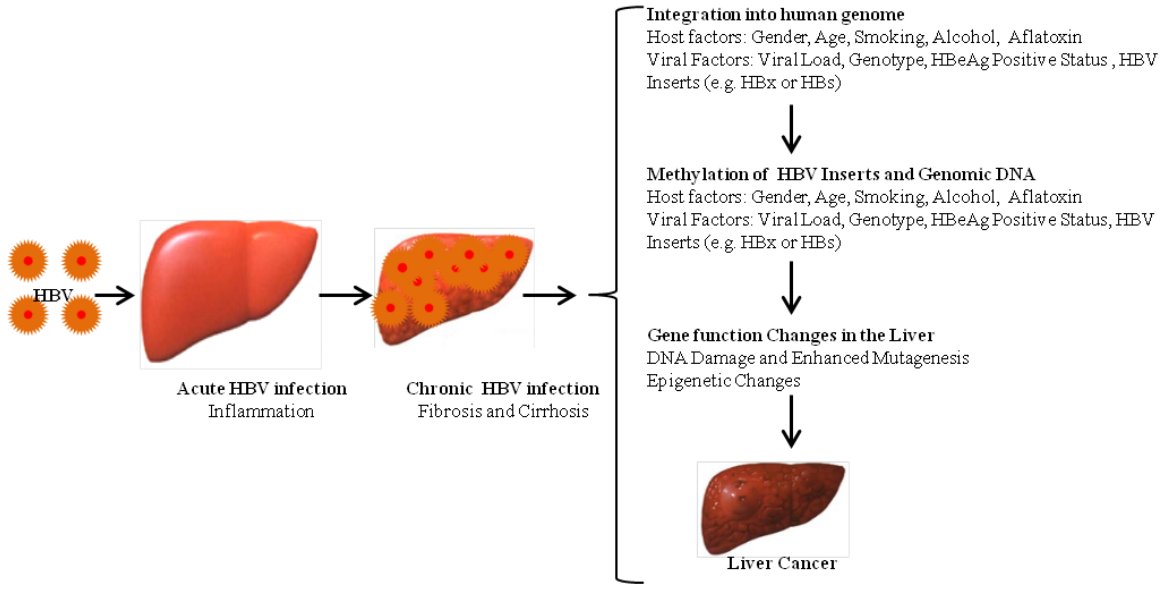

Figure 5.1: Proposed Model in which HBV Induced Genetic Alterations Contributes to Liver Disease.

Hepatitis B virus related hepatocellular carcinoma is a multi-step processes that cause hepatocyte transformation due to HBV integration into cellular DNA, epigenetic changes and transcriptional activities by hepatitis B x protein.

Abbreviations: **DNA** – Deoxyribonucleic acid, **HBV** – Hepatitis B virus, **HB eAg** – Hepatitis B virus e antigen, **HBs** – Hepatitis B virus surface antigen, **HBx** – Hepatitis B virus x gene.

5.1 DNA METHYLATION AND LIVER DISEASE

Abnormal hypermethylation of various cellular genes including host tumour suppressors has been described in various cancers and is associated with silencing of genes critical for preventing malignant transformation [25]. Gene expression has been reported in HBV infection where the DNA methylation machinery is induced as a host defence mechanism to suppress viral genes [25]. This correlates with loss of normal activity in genes important for wound-healing and immune processes. Disruption of these processes will interfere with normal cell proliferation, apoptosis and potentiates the ability to metastasize in abnormal cells as seen in chronic liver disease and malignant transformation [26 - 28].

HBV DNA integrates into the cellular DNA in liver tissue of patients with chronic HBV infection and HCC [11]. Following HBV infection, DNA methyltransferases

(DNMTs) methylate any HBV DNA integrated into the human genome. This novel epigenetic mechanism enables the suppression of HBV antigens, leading to reduced viral replication [29, 30]. HBV is thought to induce DNA methylation via HBx protein, which modulates cellular signalling pathways by activating DNMT 1 and 3 to benefit the virus. Activation of DNMT 1 and 3 inappropriately methylates host cellular genes including tumour suppressor genes whose disruption causes transformation of hepatocytes and hepatic malignancy [24, 25, 29, 30, 32].

A large group of cancer-related genes are hypermethylated in chronic HBV infection and HCC cases, and some of these were described in the literature review (see **Chapter 1**, **Section 1.4.3**). These genes include *p16^{INK4A}*, *E-cadherin*, *tumour promoter insulin-like growth factor binding 3* (*IGJBP-3*), *retinoic acid receptor beta-2* (*RAR-β2*), *glutathione-S-transferase P1* (GSTP1), *deleted in lung and oesophageal cancer 1* (*DLEC1*) and *caveolin-1* genes [32 - 37]. Hypermethylation of these genes correlates with loss of gene expression and insensitivity of malignant cells to apoptotic stimuli that leads to development tumour and progression [32 – 30].

5.2 HYPOTHESIS AND STUDY OUTLINE

The published literature reviewed in **Chapter 1 Section 4.3.3** shows that that the methylation of integrated viral DNA into the host genome is associated with the transcriptional alteration and silencing of host genes that include tumour suppressor genes critical for the development of liver cancer [30].

It was hypothesised that HBV DNA methylation may result in unintended and inappropriate methylation of adjacent host DNA. If the methylation occurred in CpG islands within gene promoter regions, this could alter the transcriptional activation of genes resulting in biological changes that are responsible for the clinical manifestations of liver disease and cancer development in chronic HBV infection.

To test this hypothesis, the presence of genome-wide methylation of promoter CpG islands in patients with chronic HBV infection was investigated using Human Promoter 1.0R array® (see **Chapter 3**). The microarray data showed the presence of genome-wide promoter region hypermethylation. This finding was validated using bisulfite DNA sequencing in patients with chronic HBV infection (see **Chapter 4**).

5.3 HBV INDUCED HEPATIC INFLAMMATION AND FIBROSIS

Hepatic stellate cells and the extracellular matrix are key sources of growth factors such as fibroblast growth factors (FGF), epidermal growth factors (EGF), tumour necrosis factor alpha (TNFα), platelet-derived growth factor (PDGF) and transforming growth factor beta (TGFβ). These growth factors control cell proliferation, differentiation and remodelling of the extracellular matrix [38 - 41]. Hepatic stellate cell activation is associated with the upregulation of cellular signalling pathways activated via transcription factors such as TGFβ, nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPK) whose targets are inflammation and fibrogenic related genes [39, 48, 50, 51, 53]. Changes in the activity of growth factors and their pathways can disrupt liver regeneration processes, resulting in the production of extracellular matrix, scarring, cirrhosis and liver cancer [38].

5.3.1 Transforming Growth Factor Beta Signalling Pathway

The *FGFRL1* gene was significantly methylated in HBV e antigen positive cases (see **Table 3.7**), *PARD6A* gene in cirrhotic HBV cases (see **Table 3.4**) and *PTOV1* gene in HBV compared to autoimmune hepatitis patients (see **Table 3.5**). In the validation work, the *FGFRL1* gene had low levels of methylation in chronic HBV infected patients (see **Table 4.2**).

The association of these genes with liver disease would be via their influence on the TGFβ signalling pathway. The TGFβ signalling pathway facilitates hepatic regeneration and wound healing processes. Alteration of these genes could mediate the mitogenic effects of TGFβ protein by increasing its fibrogenic actions in hepatic stellate cells [43, 48, 49]. TGFβ protein activates the MAPK pathway and Smad proteins which regulate the epithelial-to-mesenchymal cell transition, tumour invasion and metastasis, suggesting that these genes may have a significant role in tumour development and progression in chronic HBV infection [42, 57, 58].

In breast cancer cells, *PAR6B* gene regulates the formation of tight-junctions that mediate cell-to-cell interactions required to maintain epithelial cell polarity and accurate asymmetric cell division processes [44, 45]. Therefore, the *PARD6A* gene appears to be the essential component in the biogenesis of epithelial cell tight

junctions, suggesting that loss of its normal function may alter epithelial cell architecture, tissue remodelling and facilitate malignant transformation. In this study, *PARD6A* is hypermethylated in HBV infection (see **Chapter 3 Table 3.3**), especially in patients older than 40 years compared to normal patients (see **Chapter 3 Table 3.11**).

5.3.2 MAPK/PKC Signalling Pathways

In this study, the *CCND3* gene was highly methylated in HBV infection (see **Chapter 3**, **Table 3.3**) and in the validation experiments, this was significantly associated with an increased HBV viral load, and HBV e antigen positive status and genotype D (see **Chapter 3**, **Tables 4.4** and **4.5**)

The *CCND3* gene can be linked to liver disease through its ability to stimulate inflammation and fibrosis through retinoblastoma and MAPK/phosphatidylinositol 3 kinase (PI3K) signalling pathways [46]. MAPK in cooperation with protein kinase C (PKC) induces the transcription of cyclin, CDK 4 and 6 via the activation of transcription factors RAS, AP-1 and Myc which are recruited by PDGF growth factor. PDGF growth factor induces proliferation which when highly expressed, causes cell injury and fibrosis [46, 54, 55]. High level of *CCND3* gene expression is present in HCC occurring in cirrhotic livers and breast cancer tissues, implying that it has a significant role in the development of these cancers [46, 47].

PA2G4 protein interacts with ErbB3 protein which is associated with the epidermal growth factor receptor (EGFR). EGFR forms heterodimers with other EGF and then stimulates the intracellular tyrosine kinase activity that activates the PI3K-AKT and c-Jun N-terminal kinase (JNK)-MAPK pathways [50]. These are involved in DNA synthesis, cell proliferation and apoptosis, and their disruption will contribute to liver fibrosis and malignant transformation of cells [50]. In this study, the *PA2G4* gene which encodes PA2G4 protein was methylated in HBV patients who are HBV e antigen positive compared to normal controls (see **Chapter 3**, **Table 3.7**).

5.3.3 NF-ΚB Signalling Pathway

In this study, the *TBC1D17* gene was methylated in HBV patients who are older than 40 years compared to normal individuals (see **Chapter 3**, **Table 3.11**). It can be linked to liver disease through the NF-κB and mammalian target of rapamycin (mTOR) signalling pathways. It stimulates the activity of NF-κB and mTOR signalling pathways by binding to the auxin response factors (ARF)-GTPase activating protein (ArfGAP).

Activation of mTOR/NF-κB signalling pathways marks the activation of hepatic stellate cells and leads to regulation of tumour necrosis factor alpha (TNFα) protein [51, 53, 59, 60]. Increased expression of TNFα protein leads to the activation of transcription factors activating protein-1 and JNK. This in turn activates matrix metalloproteinase, cytokines and chemokines such as fibronectin and neutrophills which protect hepatocytes from increased ROS-mediated damage through c-myb expression [48, 50, 52]. It is possible that the *TBC1D17* gene has a protective role in the liver, and is involved in wound healing and liver regeneration processes [59, 60]. Any alteration in the transcriptional activities of this gene could disrupt the mTOR/NF-κB signalling pathway and lead to liver damage and fibrosis.

5.4 HEPATITIS B VIRAL INFECTION AND MALIGNANCY

The data obtained in this study highlights hypermethylation of CpG islands located in the promoter regions of various genes involved in a wide range of cellular signalling pathways, which when inappropriately regulated, will favour malignant transformation of hepatocytes and lead to liver cancer (see **Chapter 3**, **Table 3.12**).

5.4.1 Cell Cycle and Apoptosis

The *CCND3* and *PTGR2* genes were hypermethylated in all HBV infected cases compared to normal controls (see **Chapter 3**, **Tables 3.3**), *PARD6A* gene in cirrhotic HBV cases (see **Chapter 3**, **Table 3.4**), *NACC2* in HBV e antigen positive patients with inflammation (see **Chapter 3**, **Tables 3.7** and **3.10**) and *NAIF1* in HBV e antigen positive patients with inflammation and cirrhosis (see **Chapter 3**, **Tables**

3.7, 3.9 and **3.10**). In the validation experiments, the *CCND3* gene was highly methylated while the *NACC2* gene had low level of methylation in HBV e antigen positive patients (see **Chapter 3**, **Table 4.2**).

Hypermethylation of the identified genes reveal an important way in which HBV may inappropriately induce or inhibit apoptosis for viral replication and cell survival through the activation of HBx protein (see **Chapter 1, Section 4.3.2**). The association of these genes with HBV and liver disease may be through the activation of p53, cyclooxygenase-2, inducible nitric oxide synthase (iNOS), NF-κB, ROS, JNK, caspases 3 and 9 signalling pathways (see **Appendix 26.N**). These pathways regulate apoptosis and induction of antiviral immunity, and are critically important for hepatic inflammation, regeneration, fibrosis and HCC [61 -66]. Disruption of the normal activities of these pathways by HBx protein inhibits apoptosis leading to uncontrolled cell growth and proliferation favouring hepatocarcinogenesis [63, 64].

It is not surprising that some of the genes identified in this study are concurrently involved in regulating the cell cycle, proliferation and apoptosis as these processes are connected [67]. An intimate link between apoptotic and proliferative pathways has been demonstrated previously in several *in vivo* studies [68, 69]. These studies showed that in a large population of proliferating cells, some cells will be undergoing apoptosis and uncontrolled cell proliferation is more frequently associated with cell cycle arrest and increased or inhibited apoptosis [67 - 69].

Further research is necessary to better understand the role these genes may play in apoptosis and proliferation through the influence of HBx protein. Such studies could result in the development of therapies that inhibit HBx protein mediated activities.

5.4.2 Calcium Signalling Pathway

Microarray data analysis revealed hypermethylation of vitamin D receptor regulated genes *TRPV5* and *THRAP3*. The *TRPV5* gene was hypermethylated in HBV compared to AIH patients (see **Chapter 3**, **Table 3.4**) while *THRAP3* was hypermethylated in HBV cirrhotic patients (see **Chapter 3**, **Table 3.9**). These genes can be linked to liver disease through their association with the vitamin D receptor which plays an important role in the calcium cellular signalling pathway. The *TRPV5*

and *THRAP3* genes are epithelial calcium channels that maintain the balance of extracellular calcium by regulating 1,25-dihydroxyvitamin D3 which is the active form of vitamin D[78].

HBV infection alters cytosolic calcium signalling (see **Appendix 26.A**) as an ideal strategy to change the intracellular environment favouring viral propagation, capsids assembly and cell survival [70, 71, 74, 76]. The regulation of calcium signalling is mediated by HBx protein through the activation of transcription factor AP-1 and modulation of Pyk2 and Src kinases hepatocyte proliferation pathways for survival [72, 73]. Elevated levels of calcium signalling caused by HBx protein is required to stimulate store operated calcium (SOC), which is the entry of extracellular calcium [76]. SOC stimulates viral replication by inhibiting antiviral immunity and deregulating Pyk2/Src signalling pathways. This inappropriately regulates apoptosis and proliferation contributing to HBV pathogenesis and tumorigenesis [72 - 77].

5.4.3 Alteration of mRNA Splicing

In this study, hypermethylation of RNA processing related genes *RRN3P3, GABPB2, EXOSC3* and *SNORD48* was identified in HBV infected patients, particularly those with liver cirrhosis (see **Chapter 3, Tables 3.3, 3.4** and **3.7**). The *RRN3P3, GABPB2, EXOSC3* and *SNORD48* genes are required for the proper folding of RNA and production of protein while preventing the translation of erroneous mRNA transcripts that lack or contain premature stop codons through their degradation [81 , 82]. They also initiate RNA elongation and transcription by binding to the core promoter region through RNA polymerase I and transcriptional activation domain (TAD) [78, 79]. The relationship between these genes and HBV infection has not been investigated before. These genes could be linked to HBV infection through the transcriptional activities of HBx protein that are known to affect the mRNA surveillance pathway (see **Appendix 26.H**).

HBx protein can alter RNA splicing by binding and transactivating transcription factors through interaction with *cis*-acting elements such as RNA polymerase I and TAD binding domains (see **Chapter 3, Section 3.6.6**) [80 - 84]. HBx protein induced hypermethylation of *RRN3P3, GABPB2, EXOSC3* and *SNORD48* genes could cause

incorrect mRNA splicing and loss of protein expression or aberrant protein production, leading to severe liver disease [83, 84].

5.4.4 Protein Ubiquitination

This study has identified hypermethylation of genes encoding LNX2, UBXN1 and TRIP12 ubiquitin-related proteins in HBV infection (see **Tables 3.3, 3.4** and **3.9**). The link of LNX2, UBXN1 and TRIP12 proteins with liver disease appears to be through the ubiquitination and protein degradation pathways activated by HBx protein via the p53, Wnt and Notch signalling pathways [86, 93]. Disruption of ubiquitination and proteosomal degradation pathways by HBx protein may be required for viral evasion of the host immune defences as has been shown in Epstein-Barr viral infection [91]. LNX2, UBXN1 and TRIP12 ubiquitin-related proteins have unique domains that function as E3 ubiquitin ligases which induces ubiquitination and proteosomal degradation [92]. They are involved in the regulation of DNA repair, cell cycle, proliferation and apoptosis, which explains the possible role in causing cancers [87 - 90].

HBx protein alters the proteosomal activity of infected cells to facilitate viral entry as well as enhancing viral replication and survival [91- 93]. It protects itself from cellular degradation by manipulating and mimicking the transcriptional activity of ubiquitin-related proteins [92, 93]. It uses protein-to-protein interaction to bind to and activate ubiquitin related genes [92]. This changes ubiquitin ligase functions and inhibits protein degradation and apoptosis, contributing to malignant transformation in the liver [87 - 90].

Gene deletion mutations and methylation of ubiquitin-related genes or proteins have been observed in malignancies including HCC and are associated with loss of protein expression and normal function [87 - 90]. It is evident that they could potentially be used as tumour markers for the early detection of many malignancies including HCC.

5.4.5 Suppression of Tumour Suppressor Genes

In this study, a large group of tumour suppressor genes *GABPB2*, *NACC2*, *NAIF1*, *PA2G4*, *PARD6A*, *PATE 3*, *PCGF1*, *PTOV1*, *PTGR2*, *TUBA4A* and *TBC1D17* are hypermethylated in HBV infected liver tissues (see **Chapter 3**, **Table 3.12**). *GABPB2*, *PTGR2*, *PA2G4*, *PARD6A*, *TBC1D17*, *NAIF1*, *NACC2*, *PCGF1* and *TUBA4A* genes were particularly hypermethylated in those who were HBV e antigen positive, had cirrhosis or significant hepatic inflammation or older than 40 years (see **Chapter 3**, **Tables 3.3, 3.7, 3.9 - 3.11**). The *PTOV1*gene was hypermethylated in HBV patients when compared to AIH patients (see **Chapter 3**, **Table 3.4**) and *PATE1* gene in HCC cases (see **Chapter 3**, **Table 3.5**). In the validation experiments, *PA2G4* and *GABPB2* genes were unmethylated while *NACC2* and *TUBA4A* genes had a low level of methylation in patients who were HBV e antigen negativ (see **Chapter 3**, **Table 4.2**).

These genes can be connected to liver disease through the activation of CREB, Wnt/β-catenin, MAPK/ERK and mTOR cellular signalling pathways [94, 95]. These pathways are key targets for HBx protein. They are important regulators of cell growth or proliferation and often perturbed in liver carcinogenesis [94]. They initiate transcription by binding to CREB, APC, P1-3k, ROS, NF-κB, EBP1, ETS and ArfGab cellular transcriptional elements on the promoter regions of the downstream target genes [94 - 103]. HBV-induced hypermethylation of these genes via HBx protein could inhibit this binding and suppress transcription of genes critical for preventing tumorigenesis. In addition, loss of expression of tumour suppressor genes due to DNA methylation is a critical step in the development of malignancy in HBV infection.

5.5 CLINICAL CORRELATION OF HBV WITH METABOLISM

PTGR2 and *TRPV5* genes were highly methylated in HBV cases compared to normal controls or AIH patients (see **Chapter 3, Table 3.3**). The association of these genes with liver disease will be through their involvement in hepatic metabolism via PPARγ and calcium signalling pathways which are important in regulating hepatocyte proliferation and apoptosis (see **Appendices 26.A** and **26.L**). *PTGR2* and

TRPV5 genes regulate the metabolism of lipids, glucose and calcium [78, 106]. Disruption of these processes due to HBV induced methylation could cause hyperglycemia and fatty liver that will impair liver function [106, 107] (see **Chapter 3**, **Table 3.12**). In addition, this could disrupt type 2 diabetes mellitus signalling pathways causing insulin resistance which is often seen in chronic HBV infection and predisposes to HCC development [108, 109] (see **Chapter 3**, **Section 3.6.3** and **Appendix 26.M**).

Involvement of *PTGR2* gene in liver disease could also be through the disruption of COX-2. COX-2 is an enzyme that produces prostaglandins including PTGR2 protein and is essential for inflammation [108]. This enzyme is highly expressed in most tumours including HBV-related HCC in which it increases the expression of HBx protein which disrupts p53 mediated transcription and apoptosis by activating prostaglandin E_2 [108, 109].

5.6 ASSOCIATION OF HBV AND HIV INFECTION

This study revealed hypermethylation of *TUBA4A* and *THRAP3* genes (see **Tables 3.4** and **3.9**) which interact with HIV- 1 proteins. *TUBA4A* gene interacts with HIV-1 Protease and Gag proteins [111]. This interaction enhances the carcinogenic effect of HBx protein by disrupting microtubules formation and interferes with Bcl-2 mediated apoptosis [111].

THRAP3 protein interacts with Rev and Tat proteins [110]. The effect of HBV methylation on *THRAP3* gene is to increase viral replication by altering calcium signalling pathway and this accelerates the progression of fibrosis and cirrhosis and HCC [112 - 114].

HBV and HIV co-infection would therefore increase viral replication of both viruses through the activation of Bcl-2, MAPK and Wnt signalling pathways in a collaborative manner (see **Appendix 26.O**). These possible interactions require further investigation.

5.7 A UNIFYING HYPOTHESIS BASED ON THE LITERATURE AND THESIS RESEARCH FINDINGS

The study findings of extensive genome-wide promoter region hypermethylation in chronic HBV infected patients and the involvement of various genes whose function can be directly linked to liver disease significantly validate the study hypothesis. The inappropriate methylation could alter the transcriptional activation of genes resulting in biological changes that are responsible for the clinical manifestation of liver disease and tumour development [115 - 117]. The methylation of genes identified in this study can result in the alteration of multiple cellular processes and pathways which are known to cause liver inflammation, fibrosis, cirrhosis and cancer (see **Figure 5.2**).

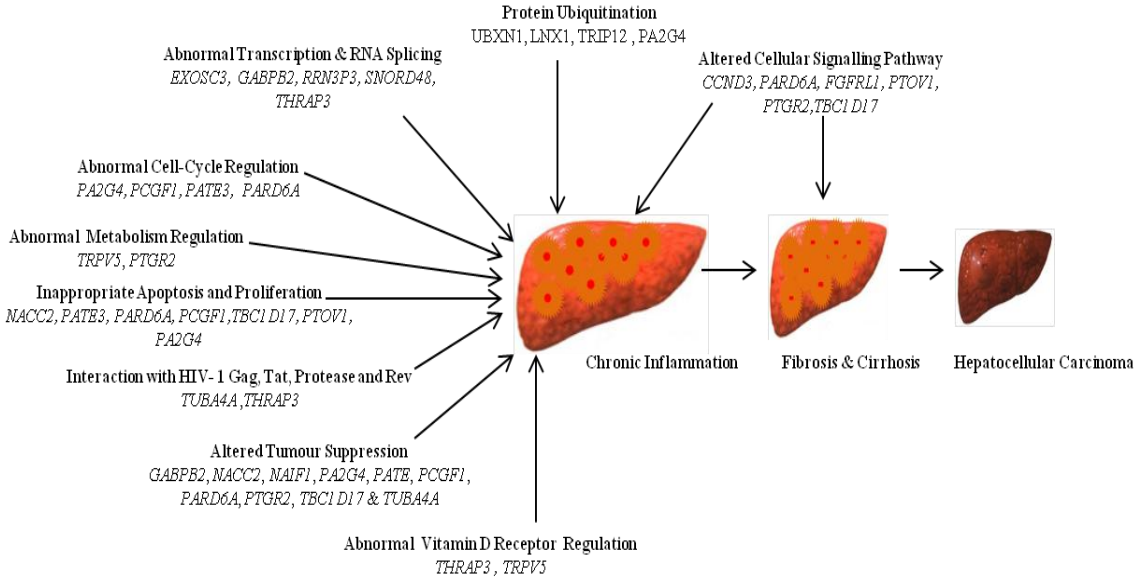

Figure 5.2: An Integrated Review of the Identified Genes and their Possible Role in Causing Liver Disease.

Multiple cellular processes and key genes or proteins are altered by HBV induced methylation and this could cause abnormalities that favour and maintain viral replication and lead to liver inflammation, fibrosis, cirrhosis and possible cancer.

HBV encodes a transcriptional activator and oncogenic protein HBx, which appears to be the main driving force behind the epigenetic alterations seen in HBV infection [118]. Abnormalities caused by the transcriptional activities of HBx protein could have

significant detrimental effects such as the suppression of antiviral immune responses and the development of pro-inflammatory responses and fibrosis. By being localised in the cytoplasm, nucleus and mitochondria of HBV infected hepatocytes, it appears that HBx protein manages to exploit the entire body of cellular signalling pathways for viral survival and propagation. HBx protein may achieve its transcriptional transactivation action by either interacting with key genes or altering their related cellular signalling pathways or by hijacking their binding partners and taking over their roles [119 -125]. These detrimental effects would connect HBV infection to malignant transformation by inducing uncontrolled cell growth, proliferation and disrupting apoptosis.

Although the underlying mechanisms are still unclear, processes such as cell cycle progression, calcium homeostasis, hepatic metabolism, protein ubiquitination, RNA splicing and vitamin D receptor regulation are key mechanisms that HBx protein alters to favour viral replication and cell survival (see **Sections 5.4.1 - 5.4.2**). Hypermethylation of a large group of tumour suppressor genes was identified in this study (see **Section 5.4.2**). Hypermethylation correlates with loss of gene expression in tumour suppressor genes as discussed in **Chapter 1, Section 1.4.3.** This could cause genetic instability, hepatocyte transformation and tumour development.

The results of the validation study on the methylation profiles of *CCND3* gene (see **Chapter 4, Section 4.4.5**) in HBV e antigen positive patients supports the hypothesis that increased methylation is associated with host and viral factors.

Further studies are needed to investigate the relationship between host factors and HBV characteristics with the severity of HBV induced DNA methylation and liver disease.

5.8 STUDY LIMITATIONS

This study had some limitations and difficulties that needed to be addressed in terms of study efficiency, costs and practicality.

a) Choice of Normal Controls

The choice of a "normal" control in microarray studies of humans is difficult as there are many causes of DNA methylation other than viral infections. Some are cumulative with age due to exposure to environmental factors such as cigarette smoking, alcohol and other toxins. Some studies are addressing this by using placental derived DNA at birth which would theoretically have pre-environmental exposure DNA damage and methylation [104]. In this study, liver derived DNA was used for microarray work. Patients who had been biopsied for the investigation of abnormal liver tests that had resolved completely and had no evidence of HBV, HCV, HIV, AIH or other causes of chronic liver disease were used as normal controls relative to chronic HBV infected cases.

To address this difficulty, a study that will investigate the presence of DNA methylation using DNA from cord blood lymphocytes is being set-up [104]. This will provide local reference DNA methylation profiles from neonatal DNA prior to environmental exposures and viral infections as has been suggested in recent studies [104].

b) Sample Size

For reasons study efficiency and costs of microarray work, the sample size used was pragmatic. The ratio of control to study cases used was 1:9, which was 2 controls to 18 HBV infected cases. This was adequate as sample size calculations had shown that a ratio of 1:4 would be adequate for this study. In the validation cohort for *CCND3* gene, the analysed sample size was limited by the success of positive colony screening and sequencing as at times more than 30 colonies may be needed before a successful insertion occurs [128]. The use of cheaper techniques that do not require the cloning step such as Methyl Profiler DNA methylation PCR array will be considered.

c) Gene Expression Data

Gene expression work which would have helped in further validation of the microarray data analysis was not done in this study due to costs. This work will be done in planned future work.

5.9 FUTURE WORK

Hypermethylation of key cellular genes identified in this study could disrupt various important processes and cellular signalling pathways that could alter hepatocyte physiology. This could contribute to mechanisms that connect HBV infection to the development of liver injury and HCC and therefore warrants further investigations.

a) Gene Expression Work

Tumour suppressor genes silencing and alteration in the cell cycle and hepatic metabolism due to DNA methylation are important in developing HBV related liver disease features such as fibrosis, cirrhosis and HCC. Methylation of *GABPB2*, *CCND3*, *NACC2*, *NAIF1*, *PA2G4*, *PARD6A*, *PATE 3*, *PCGF1*, *PTOV1*, *PTGR2*, *TUBA4A* and *TBC1D17* could result in loss of gene expression leading to liver disease. Therefore, further bisulfite DNA sequencing and gene expression studies using either microarray or real-time PCR are required to investigate the expression status of these hypermethylated genes.

b) Pathway Analysis Work

Cellular protein ubiquitination and hepatic metabolism involving calcium signalling pathway are important targets used by HBx protein to modulate transcription, proliferation and apoptosis, and benefit the virus. *TRPV5*, *THRAP3*, *LNX2*, *UBXN1* and *TRIP12* genes act as tumour suppressor genes or oncogenes, whilst in some circumstances may be targets for therapy. Methylation of these genes suggests their critical role in the development of cancer and studies investigating their role in ubiquitination and other pathways will be important as they may provide diagnostics tools and treatment possibilities.

Such studies will increase our understanding of some of the gene transcriptional changes that occur in HBV infection and provide new insights in the development of chronic HBV infection related liver disease.

c) HBV Integration into the Host Genome Work

Recent studies have now confirmed the strong correlation between high levels of HBV surface antigen and the risk of HCC [59 - 61]. This correlation is most likely to be due to an increased rate of viral surface antigen integration into the host genome which could result in higher rates of genomic DNA methylation, more gene damage and a higher risk for hepatic inflammation, fibrosis, cirrhosis and malignant transformation in these patients (see **Chapter 4, Section 4.5.2**). In view of these findings, new studies are needed that will investigate the association between HBV surface antigen levels and integration into the human genome with associated DNA methylation. These will give insight into how best to use HBV surface antigen levels in future treatment algorithms that should seek to reduce the risk of HCC as a treatment outcome.

d) Comparison of Blood and Liver Tissue Methylation Profile Work

Although the blood DNA is reliable in investigating DNA methylation profiles in patients with viral hepatitis, it is less sensitive as compared to liver tissue [134]. HBV replicates in the liver, suggesting that liver tissue could harbour a more representative methylation profile. More studies are needed to investigate whether the differences in methylation within the same patient are significantly different between blood and liver tissue derived samples.

5.10 CONCLUSIONS

The thesis findings provide new insights into the possible mechanisms by which chronic HBV infection of hepatocytes alters the cellular signalling machinery though DNA methylation, causing chronic liver disease and HBV associated liver cancer (see **Figure 5.1**). Understanding the molecular mechanisms through which the function of the genes shown to be differentially hypermethylated in this study may help in identifying possible new targets that could be used for the early diagnosis of HBV related liver complications as well as develop new therapies.

In summary, this study has successfully identified the presence of DNA hypermethylation in a cohort of HBV infected patients. The DNA methylation changes were confirmed using bisulfite DNA sequencing. Higher rates of methylation appear to be associated with an increased HBV viral load, genotype D with some genes being more hypermethylated than others, particularly the gene *CCND3*. Although the mechanisms through which particular genes are affected by HBV associated hypermethylation are not as at present entirely understood, they could be related to factors such as the HBV e antigen status, HBV surface antigen levels, the type of HBV viral insertions and a high HBV viral load [49, 50, 129].

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APPENDIX 1:

Extraction of DNA, RNA and Protein from Fresh Liver Biopsies

1. Reagents

Products	Catalog No	Local Supplier	Sources
Absolute Ethanol	E7023	Sigma, SA	Sigma, USA
95% Ethanol	E7148	Sigma, SA	Sigma, USA
TRI Reagent	T9424	Sigma, SA	Sigma, USA
1-Bromo-3-chloropropane	B9673	Sigma, SA	Sigma, USA
Isopropanol (2-propanol)	19516	Sigma, SA	Sigma, USA
Methanol	H410	Microsep, SA	Romil, USA
Sodium phosphate	S3264	Sigma, SA	Sigma, USA
Sodium hydroxide	221465	Sigma, SA	Sigma, USA
Trisodium citrate	C8532	Sigma, SA	Sigma, USA
SDS	L3771	Sigma, SA	Sigma, USA
RNA Zap	R2020	Sigma, SA	Sigma, USA
Diethylpyrocarbonate	D5758	Sigma, SA	Sigma, USA
EDTA	BB100935V	Merck, SA	Merck, Africa
1% SDS	L4522	Sigma, SA	Sigma, USA
Guanidine hydrochloride	G3272	Sigma, SA	Sigma, USA

2. Equipment

Products	Catalog No	Local Supplier	Sources
1.5ml Eppendorf tubes	071015-247	Lasec, SA	Axygen Scientific, USA
20µl, 200µl and 1000µl tips	94300120	Lasec, SA	Axygen Scientific, USA
Microcentrifuge	L0027161	Lasec, SA	Axygen Scientific, USA
Pippetman P1000, P200, P20	F123602	-	Gilson Medical Electronics, France
Heating Block	MH-4-6638	-	Fried Electric, Israel
Nanodrop Spectrophotometer	ND-1000	Promega, SA	ThermoScientific NanoDrop
Super-Mixer 220V Vortex	#288181	-	Scientific Industries, USA
VM-1000 Votex Mixer	SI-100	-	Aron Laboratory Instruments, Taiwan

PROTOCOLS

Homogenisation and Phase Separation

1. Homogenise liver tissues in 1ml of TRI Reagent solution with danca homogenizer.
2. Allow the samples to stand for 5 minutes at room temperature.
3. For phase separation, add 100µl of 1-Bromo-3-chloropropane in the homogenised tissue, cover the sample tightly and shake vigorously for 15 seconds.
4. Incubate the sample for 2 -15 minutes at room temperature.
5. Centrifuge the resulting mixture at 12000 x g for 15 minutes at 4°C.

   Note: Centrifugation separates the mixture into 3 phases: a red organic phase containing protein, an interphase containing DNA and a colourless upper aqueous phase containing RNA.
6. Transfer the aqueous phase into separate fresh tube for RNA extraction.
7. Store the interphase and organic phase at 4°C for subsequent isolation of the DNA and proteins.

RNA Extraction

1. Add 500µl of 2-propanol per into an aqueous phase sample obtained above.
2. Incubate the sample at room temperature for 5 - 10 minutes. Centrifuge at 12000 x g for 10 minutes at 4 °C. Note: The RNA precipitation will form a pellet on the side and bottom of the tube.
3. Discard the supernatant and wash the RNA pellet with 1000µl of 75% ethanol.
4. Vortex the sample and centrifuge at 7500 x g for 5 minutes at 4 °C.
5. If the RNA pellets float, repeat the wash step with 75% ethanol and centrifuge at 12000 x g and briefly dry the RNA pellet for 30 minutes by air-drying.

Note: Do not let the RNA pellet dry completely, as this will greatly decrease its solubility.

6. Add 100µl of DEPC-treated water to the RNA pellet and mix by repeated pipetting with a micropipette until dissolved.
7. Measure the absorbance with Nanodrop.
8. Store RNA samples in aliquots at -80°C.

DNA Extraction

1. Wash the DNA pellet obtained above twice in 0.1M trisodium citrate, 10% ethanol solution.

   Note: During each wash, allow the DNA pellet to stand at room temperature with occasional mixing for at least 30 minutes.

2. Centrifuge at 2000 x g for 5 minutes at 4 °C.
3. Resuspend the DNA pellet in 1.5ml of 75% ethanol and incubate the sample for 20 minutes at room temperature. **Note:** Do not to reduce the time samples remain in the washing solution. Thirty minutes is the absolute minimum time for efficient removal of phenol from the DNA.
4. Air-dry the DNA pellet for 30 minutes and dissolve in 100µl of 8 mM sodium hydroxide, pH 8 with repeated slow pipetting with a micropipette.
5. Centrifuge at 12000 x g for 10 minutes to remove any insoluble material and transfer the supernatant to a fresh eppendorf tube.
6. The purity ant integrity of DNA samples were measured with Nanodrop.
7. Store the DNA samples at -80°C in aliquots.

Protein Isolation

1. Precipitate protein from the phenol-ethanol supernatant obtained in above with 1.5ml of 2-propanol.
2. Incubate the sample at room temperature for at least 10 minutes and centrifuge at 12000 x g for 10 minutes at 4 °C.
3. Discard the supernatant and wash pellet 3 times in 2ml of 0.3M guanidine hydrochloride with 95% ethanol solution.
4. During each wash, incubate the samples in wash solution for 20 minutes at room temperature and centrifuge at 7500 x g for 5 minutes at 4 °C.
5. After the 3 washes, add 2ml of 100% ethanol and vortex the protein pellet.
6. Incubate the pellet at room temperature for 20 minutes. Centrifuge at 7500 x g for 5 minutes at 4 °C.
7. Air-dry the protein pellet and dissolve in 1% SDS.
8. Remove any insoluble material by centrifugation at 10000 x g for 10 minutes at 4°C and transfer the supernatant to a new tube.
9. Store the protein in aliquots at –80°C.

APPENDIX 2:

DNA Extraction from FFPE Tissues

1. Commercial Kits.

Products	Catalog No	Local Supplier	Sources
AllPrep DNA/RNA FFPE Kit	80234	Whitehead Scientific, SA	Qiagen, USA

2. Reagents.

Products	Catalog No	Local Supplier	Sources
Absolute ethanol	E7023	Whitehead Scientific, SA	Sigma, USA
Heptane	28471	Merck, SA	Merck, Victoria
Isopropanol (2-propanol)	19516	Sigma, SA	Sigma, USA
Methanol		Sigma, SA	Sigma, USA

3. Equipment.

Products	Catalog No	Local Supplier	Sources
1.5ml Eppendorf tubes	071015-247	Lasec, SA	Axygen Scientific, USA
20µl, 200µl and 1000µl tips	94300120	Lasec, SA	Axygen Scientific, USA
Microcentrifuge	L0027161	Lasec, SA	Axygen Scientific, USA
Pippetman P1000, P200, P20	F123602	-	Gilson Medical Electronics, France
Heating Block	MH-4-6638	-	Fried Electric, Israel
Nanodrop Spectrophotometer	ND-1000	Promega, SA	ThermoScientific NanoDrop
Super-Mixer 220V Vortex	#288181	-	Scientific Industries, USA
VM-1000 Votex Mixer	SI-100	-	Aron Laboratory Instruments, Taiwan
1.5ml Eppendorf tubes	071015-247	Lasec, SA	Axygen Scientific, USA

PROTOCOL

1. Trim excess paraffin off the samples microtome using a clean scalpel and make sure to use fresh clean scalpel for each sample.
2. Cut sections of 20µm thick and put them in 1.5ml Safe-Lock micro centrifuge tubes.
3. Immerse the sections in 500µl heptane and then incubate for 10 minutes at room temperature.
4. Add 25µl methanol and centrifuge for 2 minutes at 9000 x g after, vortexing for 10 seconds.
5. Discard all the supernatant and add 1ml absolute ethanol to the pellets.
6. Vortex the mixture and centrifuge at full speed for 2 minutes.
7. Remove all the ethanol without disturbing the pellets.
8. Leave the lid of the eppendorf tubes open and incubate at room temperature until all the ethanol has evaporated.
9. Resuspend the pellets by adding 150µl Buffer PKD and flick the tubes with a finger to loosen the pellets.
10. Add 10µl proteinase K, mix by vortexing and then incubate at 56°C for 15 minutes.
11. Cool the samples on ice for 3 minutes and centrifuge for 15 minutes at 14000 x g.
12. Carefully transfer the RNA- containing supernatants into a new eppendorf tubes and keep this for RNA purification.
13. Resuspend the DNA-containing pellets in 180µl Buffer ATL, add 40µl proteinase K, and mix by vortexing.
14. Incubate at 56°C for overnight and further incubate at 90°C for another 2 hours.
15. Briefly centrifuge the micro-centrifuge tubes to remove drops from the inside the lids.

16. Mix 200µl Buffer AL and 200µl absolute ethanol in separate eppendorf tubes and mix thoroughly by vortexing.
17. Add 400µl of the mixture to the sample and mix thoroughly again by vortexing.
18. Transfer the entire sample to a QIAamp MinElute spin columns placed in a 2ml collection tubes.
19. Centrifuge the samples for 1 minute at 13000 x g and discard the collection tubes with the flow-through.
20. Place the QIAamp MinElute spin columns in a new 2ml collection tubes.
21. Add 700µl Buffer AW1 to the spin columns and centrifuge for 15 seconds at 13000 x g to wash the spin column membranes and then discard the flow-through.
22. Add 700µl Buffer AW2 to the QIAampMinElute spin columns.
23. Centrifuge for 15 seconds at 13000 x g and then discard the flow-through.
24. Add 700µl absolute ethanol to the QIAamp MinElute spin columns and centrifuge for 15 seconds at 13000 x g.
25. Discard the collection tubes with the flow-through and place the QIAamp MinElute spin columns in a new 2ml collection tubes.
26. Open the lids of the spin columns and centrifuge at full speed for 5 minutes and then discard the collection tubes with the flow-through.
27. Place the QIAamp MinElute spin columns in a new 1.5ml collection tubes and add 60µl Buffer ATE directly to the spin column membranes.
28. Close the lids gently and incubate for 1 minute at room temperature for the pellets to become completely dissolved.

APPENDIX 3:

Kits, Products, Equipment and Consumable for DNA Microarray Work

1. Commercial kit.

Product	Catalogue Number	Supplier
QIAquick PCR Purification Kit	28104	Qiagen, USA

2. Enzymes and Ladders.

Product	Catalogue Number	Supplier
100bp Ladder		New England Biolabs MA
BstUI (comes with 100x BSA and 10x NEBuffer)	#R0518S	New England Biolabs MA
HhaI (comes with 100x BSA and 10x NEBuffer)	#R0139L	New England Biolabs MA
Human Apurinic/Apyrimidinic Endonuclease I (APE 1, comes with10X APE 1 Reaction Buffer)	78454	Affymetrix, USA
MseI (comes with 100x BSA and 10x NEBuffer)	#R0525S	New England Biolabs MA
T4-DNA ligase (10X T4 DNA Ligase Buffer with 10mM ATP)	#M0202S	New England Biolabs MA
Tag DNA Polymerase (10X Standard Tag Reaction Buffer)	#M0273L	New England Biolabs MA
Terminal DeoxynucleotidylTransferase(TdT), Recombinant (comes with 5X TdT Reaction Buffer)	72033	Affymetrix, USA
Uracil-DNA Glycosylase	71960	Affymetrix, USA

3. Washing buffers and other reagents.

Product	Catalogue Number	Supplier
Array Holding Buffer	901248	Affymetrix, USA
Control oligonucleotide B2	900301	Affymetrix, USA
DMSO	901244	Affymetrix, USA
DNA Labeling Reagent, DLR (Biotin-11-dXTP Analog)	79015	Affymetrix, USA
50Mm $MgCl_2$	#B0510A	New England Biolabs, MA
Pre-Hybridization Mix	901242	Affymetrix, USA
2X Hybridization Mix	901243	Affymetrix, USA
Stain Cocktail 1	901246	Affymetrix, USA
Stain Cocktail 2	901247	Affymetrix, USA
Wash Buffer A	900721	Affymetrix, USA
Wash Buffer B	900722	Affymetrix, USA
Array Holding Buffer	901248	Affymetrix, USA

4. Laboratory equipment.

Product	Catalogue Number	Supplier
Agarose Gel Electrophoresis Apparatus	161-3000	Bio-Rad, California, US
Eppendorf Centrifuge 5415R	54260000018	Eppendorf, Life Science 250
C1000™ Thermal Cycler	184-1000	Bio-Rad, California, US
Dry Bath Incubator	MD-01N-110	Ms Major Science, Taiwan
GeneChip®Fluidics Station 450	P/N 00-0079	Affymetrix, USA
GeneChip® Hybridization Oven 645	P/N 8001318	Affymetrix, USA
GeneChip®Probe Array (Human Promoter 1.0R array)	900775	Affymetrix, USA
GeneChip®Scanner 3000 (7G)	P/N 00-00212	Affymetrix, USA
Molecular Imager (R) Gel Doc™ XR+ with image Lab™ Software	170-8195	Bio-Rad, California, US
Vapo.Protect Mastercycler	950021209	Eppendorf, Germany
Water Bath	6032012	Thermocon, India

APPENDIX 4:

Restriction Digest of DNA with *MseI* Enzyme

Table 1: Components in the Reaction/s

Components	Volume for 1 reaction
10X NEBuffer 4	4 µl
100X BSA	0.4 µl
MseI Enzyme	1 µl
DNA	1.5 µg
Deionized water	up to a total volume of 40 µl

PROTOCOL

1. Use 1.5µg of extracted DNA for restriction digest.
2. Set up a reaction in a total volume of 40µl as shown in **Table 1**.
3. Mix well and spin down for few seconds.
4. Incubate the reaction on a heating block set at 37°C for 2 hours.
5. Load 2µl of digested product/s on 1.5% of agarose gel.

APPENDIX 5:

Purification of DNA Using Qiaquick PCR Purification Kit

PROTOCOL

1. Add 5 volume of Buffer PB to 1 volume of digested product/s and mix well.
2. Transfer the mixture into MinElute spin columns provided in the kit.
3. Centrifuge at 13000 rpm for 1 minute.
4. Discard the flow-through.
5. Wash the columns with 750µl of Buffer PE.
6. Centrifuge at 13000 rpm for 1 minute and discard the flow-through.
7. Centrifuge at the same speed for another 1 minute.
8. Transfer the MinElute Spin Columns into new eppendorf tubes and add 32µl of elution buffer to the centre of the columns.
9. Incubate for 1 minute at room temperature.
10. Elute purified DNA by centrifuging at 13000 rpm for 1 minute.

APPENDIX 6:

Annealing of Universal Linkers

Table 1: Components in the Annealing Reaction

Component	Volume for 1 reaction
10X Standard Tag Buffer	20 µl
H-12	80 µl
H-24	80 µl
Deionized water	20 µl

PROTOCOL

1. Prepare the universal linkers in a total volume of 200µl.
2. Set up the reaction as shown in **Table 1**.
3. Anneal the universal linkers in C1000^{TM} Thermal Cycler (Bio-rad, California, USA) under 95°C for 5 minutes, 70°C for 2 minutes and 52°C for 10 minutes.
4. Cool at 4°C for at least 30 minutes.

APPENDIX 7:

Ligation of DNA with Universal Linkers

Table 1: Components in the Ligation Reaction/s

Component	Volume for 1 reaction
10X T4 DNA Ligase Buffer	4 µl
H-12/H-24 Annealed Linkers	4 µl
T4 DNA Ligase	1 µl
Purified DNA	30 µl
Deionized water	5 µl

PROTOCOL

1. Set-up ligation mix as shown in **Table 1**.
2. Incubate the reaction in the water bath at 16°C for overnight.
3. Inactivate the T4 DNA ligase by incubating at 65°C for 10 minutes.
4. Run 2µl of ligated product/s on 2% agarose gel.

APPENDIX 8:

PCR Amplification of Ligated Products

Table 1: Components in the PCR Reaction/s

Component	Volume for 1 reaction	Final Concentration
10X Standard Tag Buffer	5µl	1X
10 mMdNTP + dUTP mix	2µl	200µM
10 µM *Primer B (H-24)	1.2µl	0.2µM
5 U/µl Tag Polymerase	1µl	1.25U/50µl
Ligated Product	10 µl	-
Deionized water	30.8µl	_
Total	50 µl	_

*__Primer B:__ 5'-GTTTCCCAGTCACGGTC-3'

PROTOCOL

1. Set-up PCR reactions as shown in **Table1**.
2. PCR cycle conditions: 72°C for 3 minutes, initial denaturing at 95°C for 3 minutes, 30 cycles of [further denaturing at 95°C for 30 seconds, annealing at 62°C for 1minute, elongation at 72°C for 2 minutes], 1 cycle at 72°C for 10 minutes and hold at 4°C.
3. Run 2µl of PCR products on 2% agarose gel.
4. Purify the PCR products using Qiagen PCR purification Kit as described in **Appendix 5**.
5. Use the purified PCR product/s for restriction digest in **Appendix 9**.

APPENDIX 9:

Restriction Digestion of PCR Products with *BstuI* and *HhaI* Enzymes

Table 1: Components in the Reaction/s

Components	Volume for 1 reaction
10X NEBuffer 4	4µl
100X BSA	0.4µl
BstU I enzyme	1 µl
*Hha I e*nzyme	1 µl
Purified PCR-amplified DNA	30 µl
Deionized water	3.6µl

PROTOCOL

1. Set up digestion reaction in a total volume of 40µl as shown in **Table 1**.
2. Mix well and spin down the contents in the reaction/s.
3. Place the reaction/s in C1000^{TM} Thermal Cycler (Bio-rad, California, USA) under the following cycling conditions: 37°C for 1 hour and 60°C for another 1 hour.
4. Load 2 µl of digested product/s on 1.5% of agarose gel.
5. Purify the digested product/s using Qiagen PCR Purification Kit as described in **Appendix 5**.
6. Use 10ul of purified product/s for PCR amplification as described earlier in **Appendix 8.**
7. Purify the amplified PCR product/s again as described in **Appendix 5**.
8. Use the purified PCR product/s for fragmentation protocol in **Appendix 10**.

APPENDIX 10:

Fragmentation of PCR Amplified and Purified DNA Target/s

Table 1: Components in the Reaction/s

Component	**Volume for 1 reaction**
Amplified dsDNA	7.5 µg
Deionized water	Up to 32.2 µl
10X APE I Reaction buffer	4.8µl
Uracil-DNA Glycosylase (2U/µL)	4µl
Human Apurinic/Apyrimidinic Endonuclease I (10 U/µl)	7µl
Total	48µl

PROTOCOL

1. Set-up Fragmentation mix as shown in **Table 1**.
2. Mix well and spin down the mixture.
3. Incubate the reaction/s at: 37°C for 60 minutes, 93°C for 2 minutes and 4°C for at least 2 minutes.
4. Run 2µl of PCR products on 4% agarose gel and use the rest of the products/s for labelling protocol in **Appendix 11**.

APPENDIX 11:

Labelling of Fragmented Double Stranded DNA Target/s

Table 1: Components in the Reaction/s

Components	**Volume for 1 reaction**
Fragmented dsDNA	45µl
5X Terminal DeoxynucleotidylTransferase (TdT), Recombinant Reaction Buffer	12µl
Terminal DeoxynucleotidylTransferase (*TdT*), *Recombinant* (30 U/µl)	2µl
DNA-Labelling Reagent (10mM)	1µl
Total	60µl

PROTOCOL

1. Set-up labelling mix as shown in **Table 1**.
2. Mix well and spin down the mixture.
3. Incubate the reaction/s at 37°C for 60 minutes, 70°C for 10 minutes and 4°C for at least 2 minutes.
4. Use the product/s for hybridization protocol in **Appendix 12**.

APPENDIX 12:

Hybridization of Labelled Double Stranded DNA Target/s

Table 1: Components in the reaction/s

Components	Volume for 1 reaction (µl)
Labelled dsDNA target	60µl
Control Oligonucleotides B2	3.3µl
2X Hybridization Mix	100µl
DMSO	14.0µl
Nuclease Free water	22.7µl
Total	200µl

PROTOCOL

1. Set-up hybridization mix as shown in **Table 1**.
2. Flick-mix the reaction/s and spin down the mixture.
3. Incubate the reaction/s at 99°C for 5 minutes and 45°C for 5 minutes.
4. Centrifuge at 14000 rpm for 1 minute.
5. Inject 200µl hybridization cocktail into the GeneChip® Human Promoter 1.0R probe arrays.
6. Hybridize the probe arrays for 16 hours in 45°C GeneChip® Hybridization Oven 645 set at 60 rpm. (**Note**: Hybridization oven must be switched on and set at the required conditions at least 30 minutes before use).
7. After hybridization, transfer the hybridization mixture from probe arrays into fresh tubes and store at -80°C for future use.
8. Inject in 200µl of Array Holding Buffer into the probe arrays.
9. Use the probe arrays for washing and staining protocol in **Appendix 14**, after priming protocol in **Appendix 13**.

APPENDIX 13:

Registration of Probe Arrays and Preparation of GeneChip® Fluidics Station 450/250 Protocol

PROTOCOL

Step 1: Registering of Probe Arrays

1. Prior to washing and staining, the arrays were first registered in Affymetrix GeneChip® Command Console (AGCC) that operates GeneChip® Fluidics Station 450/250.

Step 2: Preparation of the Fluidics Station

1. Switch on the GeneChip® Fluidics Station 450 that is operated using AGCC connected on the computer.
2. Click **AGCC Fluidics Control** icon in the **AGCC Launcher** and then click on **Master/Station controls**.
3. Select **Probe Array Type** (e.g. **Human Promoter 1.0 R array**).

Step 3: Priming the Fluidics Station

1. This step is done only when the fluidics station is first started or when wash solutions are changed. It is important to ensure that the lines of the GeneChip® Fluidics Station 450 are filled with correct buffers and that the GeneChip® Fluidics Station 450 is ready for running the staining and washing protocol.

Table 1: Washing Buffers for Priming Protocol

Buffers	Volume
Wash Buffer A	300ml
Wash Buffer B	300ml
Deionised water	300ml

2. Fill the intake buffer reservoirs on the right side of the instrument with deionised water, appropriate Wash A and Wash B solutions in the order set in **Table 1**.
3. Empty the waste bottle reservoir and fill the water reservoir with deionised water.
4. Place 3 empty 1.5ml microcentrifuge vials into stain holder position 1, 2 and 3.
5. Press the washblock lever into the engaged or closed position and push the needle lever into the down position.
6. Select **Protocol** .e.g. **Prime _450 maintenance protocol** and from the **AGCC Fluidics Control**.
7. Select **Station ID** and this will allow selection of the number of modules to use **e.g.** if 4 arrays are processed, all 4 modules must be selected by clicking **Run All Modules on Current Station** or by clicking **Run All Modules on Current Station button**.
8. When the priming protocol is complete, the LCD window displays the message **Ejects & Inspects cartridge**.
9. Continue with staining and washing protocol of probe arrays in **Appendix 14**.

APPENDIX 14:

Staining and Washing of the Probe Arrays using GeneChip® Fluidics Station 450/250

Table 1: Preparation of Staining Reaction/s

Reagents	Volume	Eppendorf Tubes
Stain Cocktail 1 (Light sensitive, so need to be covered with foil)	600µl	1
Stain Cocktail 2	600µl	2
Array holding buffer	800µl	3

PROTOCOL

1. Gently tap the bottles of reagents to mix well before use.
2. Aliquot each staining reagents and Array Holding Buffer into individual l.5ml eppendorf tubes as shown in **Table 1**.
3. Spin down all vials to remove the bubbles.
4. Check on the GeneChip® Fluidics Station 450/250 whether the priming protocol from **Appendix 13** is complete. If it is complete, the LCD window will display the message **Ejects & Inspects cartridge**.
5. Insert the appropriate probe array into the designated module of the GeneChip® Fluidics Station 450 while the cartridge lever is in the down or eject position.
6. Make sure the wash solutions and water reservoirs contain enough liquid for the run to complete and that the waste bottle has enough room to collect waste from the running protocol.
7. Remove three 1.5ml microcentrifuge vials put into stain holder position 1, 2 and 3 during priming protocol.
8. Follow the instructions on the LCD window of the GeneChip® Fluidics Station 450 by

placing the three microcentrifuge vials with reagents aliquots as shown in **Table 1** into the sample holders 1, 2, and 3 on the fluidics station.

a) Place one vial containing 600µl Stain Cocktail 1 containing SAPE Stain Solution in sample holder 1.

b) Place one vial containing 600µl Stain Cocktail 2 containing Antibody Solution in sample holder 2.

c) Place one vial containing 800µl of Array Holding Buffer in sample holder 3.

9. Press down the needle lever to snap needles into position and start the run.
10. The run begins and the Fluidics Station dialog box at the workstation terminal and the LCD window will display the status of the washing and staining as the protocol progresses.
11. When the protocol is complete, the LCD window displays the message **Ejects & Inspects cartridge**.
12. Remove the probe arrays from the GeneChip® Fluidics Station 450 modules by first pressing down the cartridge lever to the eject position and continue with scanning protocol in **Appendix 15**.
13. If there are no more samples to wash and stain, shut down the fluidics station by performing the **Shutdown Protocol** as follows:

    a) Place the Wash A and Wash B lines into deionized water.

    b) Place empty 1.5ml microfuge tubes in the stain holders.

    c) Run the **Shutdown_450 Protocol.**

14. When the shutdown protocol is complete, the LCD display will indicate that GeneChip® Fluidics Station 450 is no longer primed. Turn off the GeneChip® Fluidics Station 450/250 using the toggle switch on the lower left side of the machine.

APPENDIX 15:

Scanning of the Probe Arrays Protocol

PROTOCOL

1. Turn on GeneChip® Scanner 3000 (7G) 30 minutes before use. This instrument was operated by GeneChip® operating software version 1.4.
    a) Soon after turned on, both the yellow and green lights of the scanner will be switched on and the scanner will enter the laser warm-up state. After about 10 minutes, the green light will turn off and the yellow light will remain on.
    b) Wait 10 minutes for the laser to stabilize. When the green light turns on, the scanner is ready to scan an array.
2. Check if there are bubbles in the probe arrays before scanning.
    a) If there are no bubbles, proceed to scanning protocol in the GeneChip® Scanner 3000 (7G).
    b) If you see bubbles in the probe arrays, pipette in and out the **Array Holding Buffer** until the bubbles are disappeared and then proceed to scanning protocol.
3. On the AGCC control scan, click **Start button.**
4. On the scanner dialog box, select the **Sample Name** of the array you wish to scan from **Sample File Name** drop-down list.
5. After selected the **Sample Name**, click **Start** in the start scanner dialog box to start the run. After clicking **Start** the scanner dialog box will appear, and the scanner door will open with the chip transport mechanisms rises to accept the probe array.
6. Gently clean access fluid from around the septa of probe array with soft cloth and load the probe array in the chip transport mechanism and click **OK** in the scanner dialog box to start scanning process.

7. After scanning process is complete, AGCC will save the image data, generates "**.CEL**" files and ejects the probe array. If no more probe array to scan, shut down the AGCC control scanner and switch off the scanner.

Appendix 15: Scanning of the Probe Arrays Protocol

APPENDIX 16:

Commercial Kits, Reagents and other Consumables Used For Isolation of DNA from Blood and Bisulfite Sequencing Work

1. Commercial Kits

Product	Catalogue Number	Local Supplier	Supplier
EZ DNA Methylation-Gold™ Kit	D5006	Ingaba Biotec, SA	Zymo Research, Germany
pGEM-T Easy Vector System II [comes with a vector, JM109 Cells, T4 DNA Ligase and Buffer]	A1380	Whitehead Scientific, SA	Promega, USA
QIAquick Gel Extraction Kit	28704	Whitehead Scientific, SA	Qiagen, USA
Wizard® Genomic DNA Purification Kit	1125	Anatech	Promega, USA

2. Restriction Enzymes and Primers

Product	Catalogue Number	Local Supplier	Supplier
100bp Ladder		Ingaba Biotec, SA	New England Biolabs MA
BamHI FastDigest Enzyme, comes with 10X FastDigest and FastDigest Green Buffers	FD0054	Ingaba Biotec, SA	Thermo Scientific, USA
250U HotStarTaq DNA Polymerase, comes with PCR buffer, 2Q solution and 25mM Magnesium Chloride ($MgCl_2$)	203203	Whitehead Scientific, SA	Qiagen, USA
pUC/M13 Forward Primer	#Q5601	Whitehead Scientific, SA	Promega, Madison, USA
pUC/M13 Reverse Primer	#Q5421	Whitehead Scientific, SA	Promega, Madison, USA
Primers for Bisulfite DNA Sequencing	-	Whitehead Scientific, SA	Promega, Madison, USA

3. Preparation of Buffers

Buffer	Composition
EDTA	0.1M EDTA, deionised water, Autoclave
TBE (10X)	0.9M Tris base, 0.09M Boric Acid and 0.02M Ethylenediaminetetraacetic acid (EDTA)[pH 8.0]
Tris buffer	1M Tris Base and deionised water [pH 8.0], Autoclave

4. Bacterial Culture Additives

Product	Catalogue Number	Local Supplier	Supplier
-Bromo-4-chloro-3-indolyl b-D-galactopyranoside (X-gal)	72035	Sigma, SA	Sigma, USA
Ampicillin Sodium Crystaline	A9518	Sigma, SA	Sigma, USA
Isopropyl B-D Thiogalactopyranoside Diox (IPTG)	16758	Sigma, SA	Sigma, USA
5-Bromo-4-chloro-3-indolyl b-D-galactopyranoside (X-gal)	72035	Sigma, SA	Sigma, USA
Ampicillin Sodium Crystaline	A9518	Sigma, SA	Sigma, USA

5. Equipment

Products	Catalogue Number	Local Supplier	Sources
0.2ml and 0.5 ml PCR tubes	93400150	Lasec, SA	Axygen Scientific, USA
1.5ml Eppendorf tubes	071015-247	Lasec, SA	Axygen Scientific, USA
20µl, 200µl and 1000µl tips	94300120	Lasec, SA	Axygen Scientific, USA
Microcentrifuge	L0027161	Lasec, SA	Axygen Scientific, USA
C1000^{TM} Thermal Cycler	184-1000	-	Bio-Rad, California, US
Pippetman P1000, P200, P20	F123602	-	Gilson Medical Electronics, France
90mm Petri Dish, Single Vent	90032	Whitehead Scientific, SA	Qiagen, USA
10µl Inoculating Loop, Hard, Dark Blue	SL10H	Whitehead Scientific, SA	Qiagen, USA
Heating Block	MH-4-6638	-	Fried Electric, Israel
Nanodrop ND-1000 UV-Vis Spectrophotometer	844-0020xx	Promega, SA	ThermoScientific, USA
Super-Mixer 220V Vortex	#288181	-	Scientific Industries, USA
VM-1000 Votex Mixer	SI-100	-	Aron Laboratory Instruments, Taiwan
Water Bath	SMC ETech	-	Thermocon

6. Chemicals and other Laboratory Consumables

Product	Catalogue Number	Local Supplier	Supplier
Agar	A7002	Sigma, SA	Sigma, USA
Deoxynucleotide Triphosphate Set	DNTP100	Sigma, SA	Sigma, USA
Sodium Chloride	S9888	Sigma, SA	Sigma, USA
Tryptone	T9410	Sigma, SA	Sigma, USA
Yeast Extract	70161	Sigma, SA	Sigma, USA

APPENDIX 17:

Isolation of Genomic DNA from Blood Samples Using Wizard® Genomic DNA Purification Kit

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PROTOCOL

1. To 2.5ml blood sample, add 7.5ml of Cell Lysis Solution in a sterile 15ml centrifuge tubes.
2. Gently invert the tubes 5-6 times to mix thoroughly and incubate the mixture at room temperature for 10 minutes to lyse the red blood cells. Invert intermittently during the incubation at least 2-3 times.
3. Centrifuge at 2000 x g for 10 minutes at room temperature.
4. Decant and remove as much supernatant as possible without disturbing the visible white pellet (**Note**: If the blood has been frozen, repeat the above steps until the pellet is nearly white. There may be some loss of DNA in frozen samples. If the pellet appears to contain only red blood cells, add an additional aliquot of Cell Lysis Solution and repeat above steps).
5. Vortex the tubes vigorously for 10-15 seconds to re-suspend the white blood cells. The white blood cells need to be re-suspended to obtain efficient cell lysis.
6. Add 2.5ml of Nuclei Lysis Solution, pipet the solution 5-6 times to lyse white blood cells. The solution should become very viscous. (If clumps are visible after mixing, incubate the solution at 37°C until the clumps are disrupted. If clumps are still visible after 1hr, add 3ml of additional Nuclei Lysis Solution and repeat the incubation).
7. **Optional:** Add RNase A to a final concentration of 20μg/ml, to the nuclear lysate and mix the samples by inverting the tubes 25 times. Incubate the mixture at 37°C

for 15 minutes and then cool to room temperature.

8. Add 825µl of Protein Precipitation Solution to the nuclear lysate and vortex for 10-20 seconds vigorously. Small protein clumps may be visible after vortexing. (If additional Nuclei Lysis Solution was used in Step 6 above, additional 825µl of Protein Precipitation Solution should be used).
9. Centrifuge at 2 000 x g for 10 minutes at room temperature. A dark brown protein pellet should be visible.
10. Transfer the supernatants to a 15ml centrifuge tubes containing 2.5ml of isopropanol at room temperature.
11. Gently mix the solution by inversion until the white thread-like strands of DNA form a visible mass.
12. Centrifuge at 2 000 x g for 1minute at room temperature. The DNA should be visible as a small white pellet.
13. Decant the supernatants and add 10ml of room temperature 70% ethanol to the DNA. Gently invert the tubes several times to wash the DNA pellet and the sides of the tube. Repeat the centrifugation step at 2 000 x g for 1 minute at room temperature.
14. Carefully aspirate the ethanol to avoid aspirating the DNA pellet into the pipette. Air-dry the DNA pellet for 10-15 minutes.
15. Add 207.5µl of DNA Rehydration Solution to the tubes and re-hydrate the DNA by incubating at 65°C for 1 hour. Alternatively, re-hydrate the DNA by incubating the solution overnight at room temperature or at 4°C.
16. Store the DNA in aliquots at 2-8°C.

APPENDIX 18:

Restriction digests of Genomic DNA with *BamHI* Enzyme

Table 1: Components in the Reaction/s

Components	Volume for 1 reaction
10X FastDigest Buffer	3µl
BamHI FastDigest Enzyme	1µl
Patient genomic DNA	2µg
Deionised water	Up to a total volume of 30µl

PROTOCOL

1. Pipette 2µg of genomic DNA in 0.2ml PCR tube.
2. Set up the reaction/s in a total volume of 30µl as shown in **Table 1**.
3. Mix well and spin down the reactions for few seconds.
4. Incubate the reactions on a water bath at 37°C for 15 minutes.
5. Inactivate the enzyme at 80°C for 5 minutes.
6. Load 10µl of digested product/s on 1.2% of agarose gel.
7. Use 20µl for bisulfite DNA conversion in **Appendix 19**.

APPENDIX 19:

Bisulfite Conversion of Digested DNA using EZ DNA Methylation-Gold™ Kit

PROTOCOL

1. Prepare CT conversion reagent by adding 300µl of M-Dilution Buffer, 50µl M-Dissolving Buffer into CT conversion reagent tube provided in the kit.
2. Add 130µl of CT conversion reagent into 20µl of the *BamHI* digested genomic DNA recovered in **Appendix 18**.
3. Perform the following temperature steps: 98°C for 10 minutes, 50°C for 16 hours, 4°C for 10 minutes.
4. Add 150µl of CT converted DNA into Zymo-Spin™ IC Column with 600µl of M-Binding Buffer and mix by inverting several times with the cap closed.
5. Centrifuge at 14000 x g for 30 seconds and discard the flow-through.
6. Add 100µl of M-Wash buffer with 100% ethanol to the column and centrifuge again at the same conditions.
7. Add 200µl of M-Desulphonation Buffer to the column and incubate at room temperature for 20 minutes with frequent mixing by vortexing.
8. Add 200µl of M-Wash buffer with 100% ethanol to the column and centrifuge as previously described. Repeat this step one more time.
9. Throw the tube with flow-through and place the Zymo-Spin™ IC Column into a new 1.5ml eppendorf tube.
10. Elute the converted DNA by spinning at 14000 x g for 30 seconds.
11. Use 150ng of bisulfite converted DNA for PCR amplification in **Appendix 20**.

APPEDNDIX 20:

PCR Amplification of Bisulfite Converted DNA

Table 1: PCR Components for Bisulfite Converted DNA

Components	**Volume for 1 reaction**	**Final Concentration**
10X PCR Tag Buffer	5µl	1X
10mM dNTP	1µl	200µM
10µM Forward Primer	2.5µl	0.5µM
10µM Forward Primer	2.5µl	0.5µM
250U HotStar Taq Polymerase	0.25µl	2.5U/ reaction
Bisulfite converted DNA	150ng	150ng
Deionized water	Up to 50µl	_
Total	50µl	_

PROTOCOL

1. Set-up the PCR reactions as shown in **Table 1**.
2. PCR cycle conditions: 1 cycle of initial denaturation of 95°C for 15 minutes, 5 cycles of [denaturing at 94°C for 45 seconds, annealing at a temperature predicted by primer designer software for 45 seconds, elongation at 72°C for 90 seconds], 5 cycles of [denaturing at 94°C for 45 seconds, annealing at 5°C less temperature predicted by primer designer software for 45 seconds, elongation at 72°C for 90 seconds], 35 cycles of [denaturing at 94°C for 45 seconds, annealing at a primer-specific annealing temperature for 45 seconds, elongation at 72°C for 90 seconds], 1 cycle of final extension at 72°C for 5 minutes and hold at 4°C.
3. Run 5µl of the PCR product/s on 1.2% agarose gel.
4. Excise the product/s from gel and weigh the excised product/s.
5. Purify the excised product/s using QIAquick Gel Extraction Kit as described in **Appendix 21**.

APPEDNDIX 21:

Purification of Excised Gel Product/s using QIAquick Gel Extraction Kit

PROTOCOL

1. Add 5 volume of Buffer PB to 1 volume of amplified PCR or excised gel product/s and mix well.
2. Transfer the reaction mixtures into MinElute spin columns provided in the kit.
3. Centrifuge at 13000 rpm for 1 minute.
4. Discard the flow-through.
5. Wash the columns with 750µl of Buffer PE with 100% ethanol.
6. Centrifuge at 13000 rpm for 1 minute, discard the flow-through and further centrifuge at the same speed for another 1 minute.
7. Transfer the MinElute Spin Columns into new eppendorf tubes and add 32µl of elution buffer

   to the centre of the columns. Incubate for 1 minute at room temperature.
8. Elute the purified DNA by centrifuging at 13000 rpm for 1 minute.
9. Use the purified product/s for ligation in **Appendix 22**.

APPENDIX 22:

Cloning of Purified DNA into pGEM-T® Easy Vector System II

Table 1: Components for Ligation Reaction/s

Component	Standard Reaction	Positive Control	Background Control
2X Rapid Ligation Buffer, T4 DNA ligase	5µl	5µl	5µl
pGEM-T Easy vector (50ng)	1µl	1µl	1µl
Purified DNA	3µl	-	-
Control Insert	-	2µl	-
T4 DNA Ligase	1 µl	1µl	1µl
Nuclease-free water to a final volume of	10µl	10µl	10µl

PROTOCOL

1. Determine the amount of insert DNA required using Biomath calculator sourced at www.promega.com/biomath.
2. Centrifuge pGEM-T® Easy vector and insert DNA tubes to collect the contents at the bottom of the tubes in 0.5ml PCR tubes.
3. Shake the 2X rapid Ligation buffer vigorously before each use.
4. Set ligation reactions as described in **Table 1**.
5. Mix the reactions by pipetting and incubate overnight at 4°C.

APPEDNDIX 23:

Preparation of Media, Plates and Transformation of Ligated Products

Table 1: Preparation of Buffers and Bacterial Culture Media used in this Study

Buffer	**Composition**
Ampicillin [100µg/ml]	0.2 g Ampicillin in 10ml deionised water, filter sterilize and store the aliquots at -20°C
EDTA	0.1M EDTA, deionised water, Autoclave
IPTG [238.3µg/ml]	0.238g IPTG in 10ml deionised water, filter sterilize and store the aliquots at -20°C
TBE (10X)	0.9M Tris base, 0.09M Boric Acid and 0.02M Ethylenediaminetetraacetic acid (EDTA)[pH 8.0]
Tris buffer	1M Tris Base and deionised water [pH 8.0], Autoclave
2xYT medium	1% Tryptone, 0.5% Yeast Extract, 1% Sodium Chloride [pH7.0], Autoclave
2xYT Agar	1% Tryptone, 0.5% Yeast Extract, 1% Sodium Chloride and 3% Agar, Autoclave
X-gal [200µg/ml]	0.2g X-gal in 10ml dimethyl sulfoxide (DMSO), filter sterilize, cover with aluminium foil and store the aliquots at -20°C

PROTOCOL

1. For each ligation reaction, prepare two 2xYT agar plates as described in **Table 1**.
2. Supplement the agar with 100µg/ml ampicillin, 100µg/ml X-gal and 200µg/ml IPTG.
3. Remove J109 competent cells out of the freezer and thaw on ice for 5 minutes.
4. Add 10µl of ligated products into 1.5ml eppendorf tubes on ice.
5. Carefully transfer 50µl of cells into the tube with ligated products and mix gently by flicking the tube with a finger. Use 100µl cells for determination of transformation efficiency
6. Incubate on ice for 20 minutes.
7. Heat-shock the cells for 50 seconds at 42°C in water bath.
8. Immediately put the cells on ice for 2 minutes to recover.
9. Add 940µl of 2xYT media to the cell transformed with ligated products.
10. Shake at 37°C for 1.5 hours.

11. Spread 100µl of cells transformed with ligated products onto 2xYT plates with X-gal, IPTG and ampicillin.
12. Centrifuge the remaining cells at 1000 x g for 10 minutes, resuspend the pellet in 200µl of 2xYT medium and plate 100µl for higher number of colonies.
13. For transformation efficiency, perform 1:10 dilution with 2xYT and plate 100µl.
14. Incubate the plates up to 24 hours at 37°C.

APPENDIX 24:

Screening Positive Clones with Vector and Inserts Using Colony PCR

Table 1: Components in the Reaction/s for Colony PCR

Components	Volume for 1 reaction	Final Concentration
10X PCR Go Tag Buffer	5µl	1X
10mM dNTP	2µl	200µM
10µM *Forward Primer	1µl	0.2µM
10µM *Reverse Primer	1µl	0.2µM
250U HotStar Taq Polymerase	1.25µl	1.25U/50µl
Colony Supernatant	3µl	_
Deionized water	36.8µl	_
Total	50µl	_

***pUC/M13 Forward Primer**: 5'-GTTTTCCCAGTCACGAC-3'
* **pUC/M13 Reverse Primer**: 5'-CAGGAAACAGCTATGAC-3'

PROTOCOL

1. The selection of colony is based on blue/white screening method. Clones with no PCR products usually produce blue colonies and with PCR products produce white colonies.
2. Pick up 5 white colonies from plate/s plated with cells transformed with each ligated reaction.
3. Resuspend the colonies individually into 10µl of deionised water and heat at 95°C for 5 minutes.
4. Centrifuge at 10000 x g for 1 minute and keep the supernatant.
5. Set-up PCR mix as shown in **Table 1**.
6. PCR cycle conditions: 1 cycle of denaturing at 95°C for 2 minutes, 35 cycles of [denaturing at 95°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute], 1 cycle of 72°C for 5 minutes and hold at 4°C.

7. Run 5µl of PCR product/s on 1.2% agarose gel.
8. Purify the PCR product/s using Qiagen Gel Extraction Kit as described in **Appendix 21**.

APPENDIX 25:

DNA Sequencing of Colony PCR Products and Data Analysis

PROTOCOL

A. DNA Sequencing

1. Sent positive colony PCR products of about 8ng/µl each for sequencing in the Central Sequencing Facility at the University of Stellenbosch, South Africa.
2. Use of BigDye Terminator V3.1 sequencing kit® (Applied Biosystem) for DNA sequencing following manufacture's protocol with slight modifications.
3. Sequence the DNA using 1pmol/µl of both M13 forward (-20) 5'- GTA AAA CGA CGG CCA GT-3' and M13 reverse (-27) 5'- CAG GAA ACA GCT ATG AC-3' primers (Whitehead Scientific, SA**)**.
4. After sequencing, extract sequencing results in FASTA format using a trace file viewer like Chromas[1]**.**

B. Sequence Alignment

1. Use of BioEdit Sequence Alignment Editor to create ClustalW multiple alignment of the gene promoter region sequence downloaded from Ensembl[2] and the bisulfite converted sequence of positive clones.

C. Analysis of Demographic and Clinical Data

1. Analyse the demographic and clinical data of the study patients using STATA 13 (College Station, Texas, USA).

2. Summarize categorical binary data as counts and percentages and categorical ordinal as median and inter-quantile values.
3. Summarize ordinal numerical data as median and inter-quantile values and continuous numerical data as means and standard deviations.
4. Performed Pearson Chi-Squared test and Fisher's exact test to investigate possible association between independent categorical variables.

REFERENCES

1. Trace file viewer like Chromas. http://www.technnelysium.com.au. Assessed at April 2013.
2. Ensembl Genome Browser. http://www.ensembl.org/index.html. Accessed April 2013.

APPENDIX 26:

Pathway Analysis Charts

Partek Genomic Suite software was used to investigate the biological significance of genes in each microarray analysis group using the GO terms. Some of these pathways are outlined below as:

A. HBV Infection and HCC Calcium Signalling Pathway

B. HBV Infection and TGF Beta Pathway

C. HBV Infection and TB Pathway

D. HBV Infection and Cancer Pathway

E. HBV Infection and Cell Adhesion Pathway

F. HBV Infection Pathway

G. HBV Infection and Tight Junction Pathway

H. HBV Infection and mRNA Surveillance Pathway

I. HBV Infection and MAP Kinase Pathway

J. HBV Infection and the Cell Cycle Pathway

K. HBV Infection and Viral Carcinogenesis Pathway

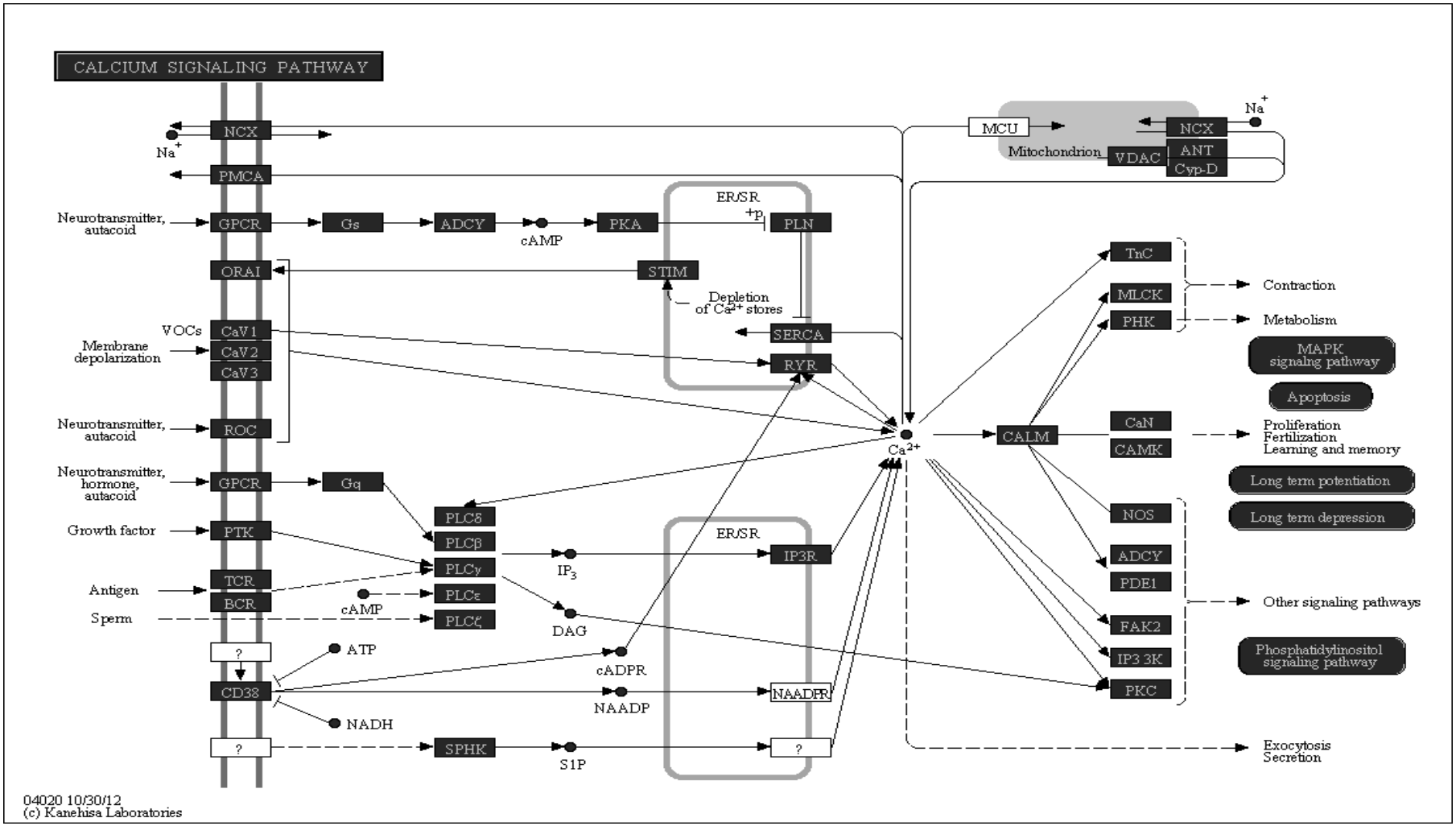
L. HBV Infection and PPAR Gamma Signalling Pathway

M. HBV Infection and Type 2 Diabetes Mellitus Signalling Pathway

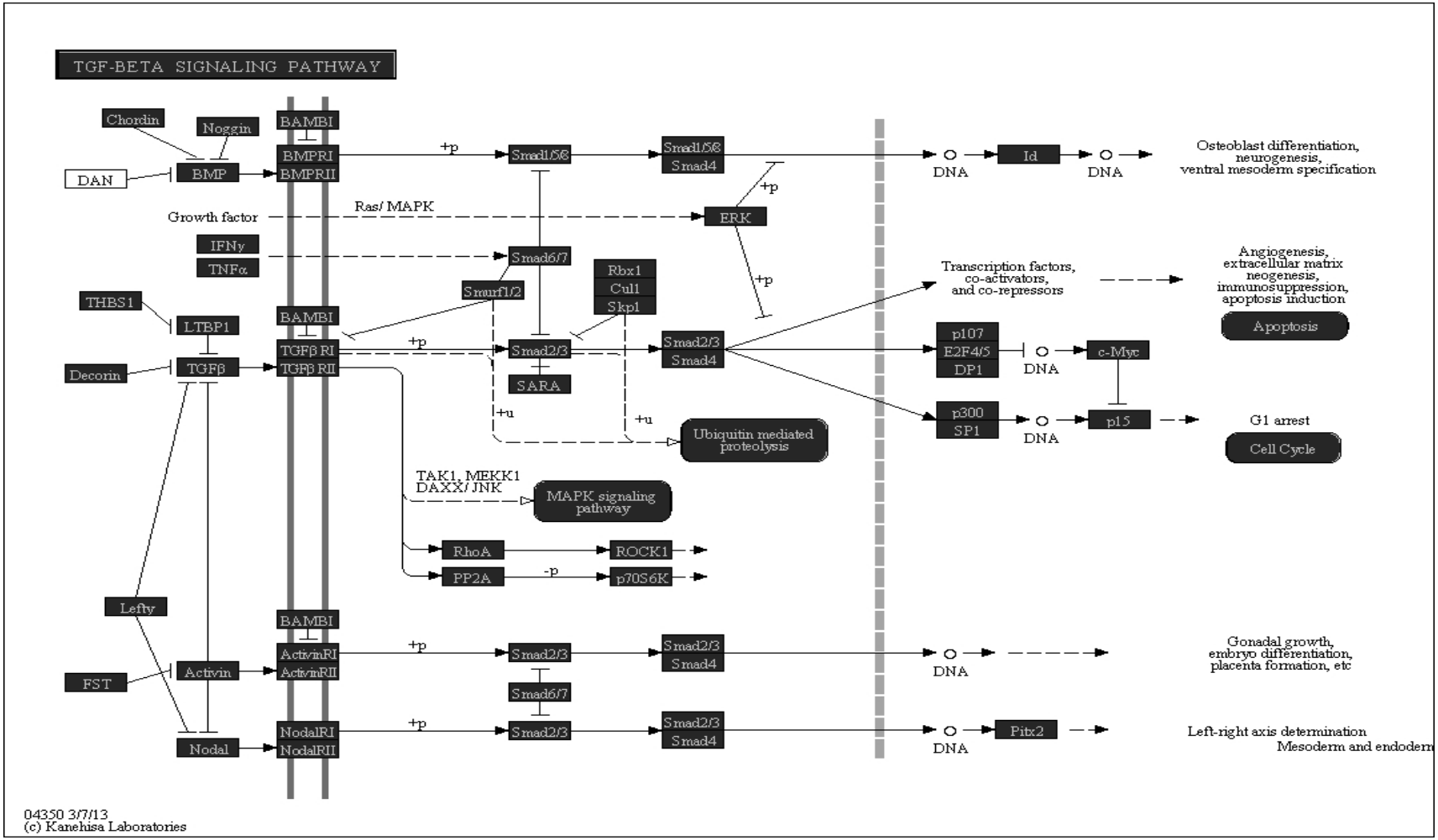
N. HBV Infection and Apoptosis Pathway

O. HBV Infection and HTLV-1 Infection Pathway

A. HBV Infection and HCC Calcium Signalling Pathway

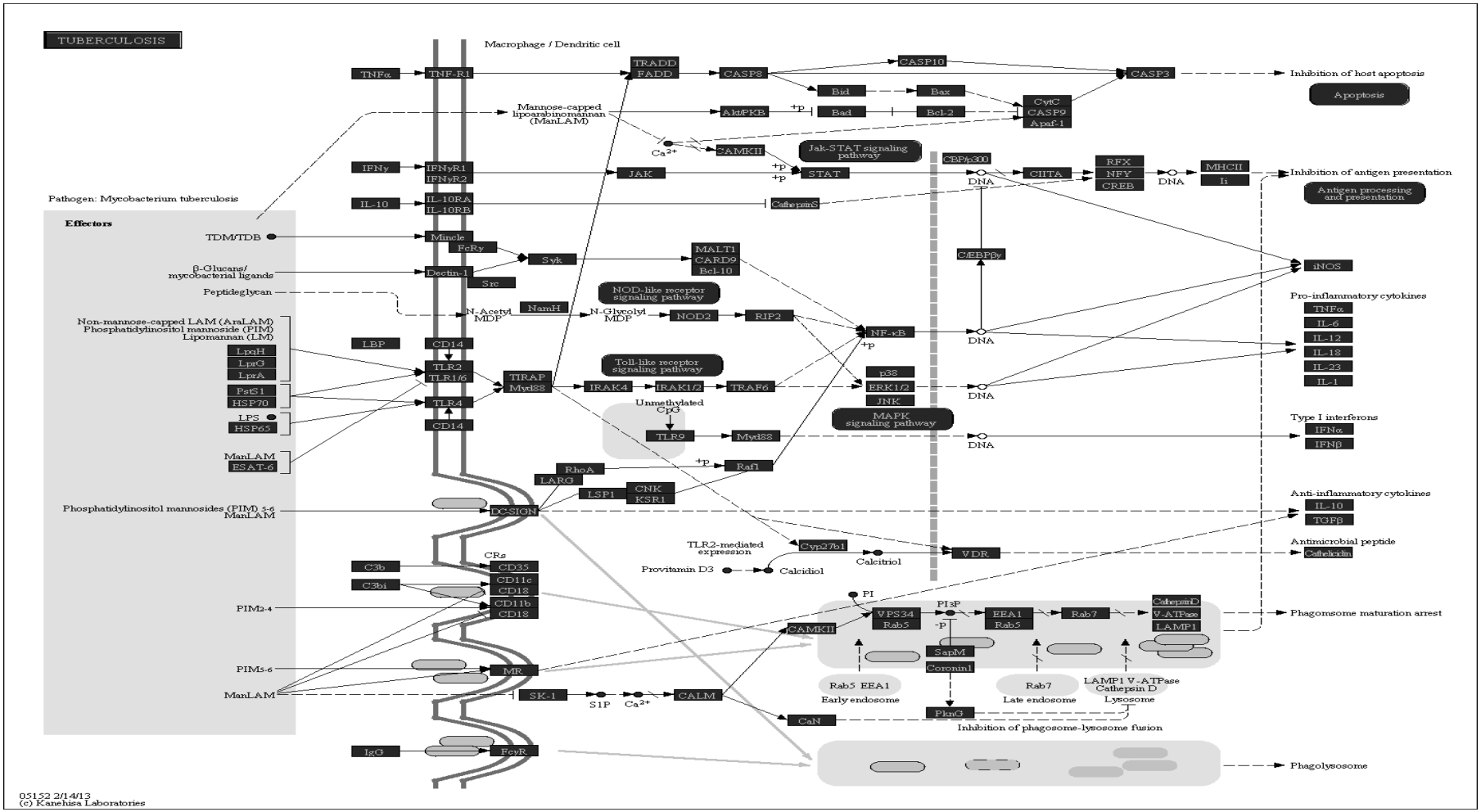


B. HBV Infection and TGF Beta Pathway



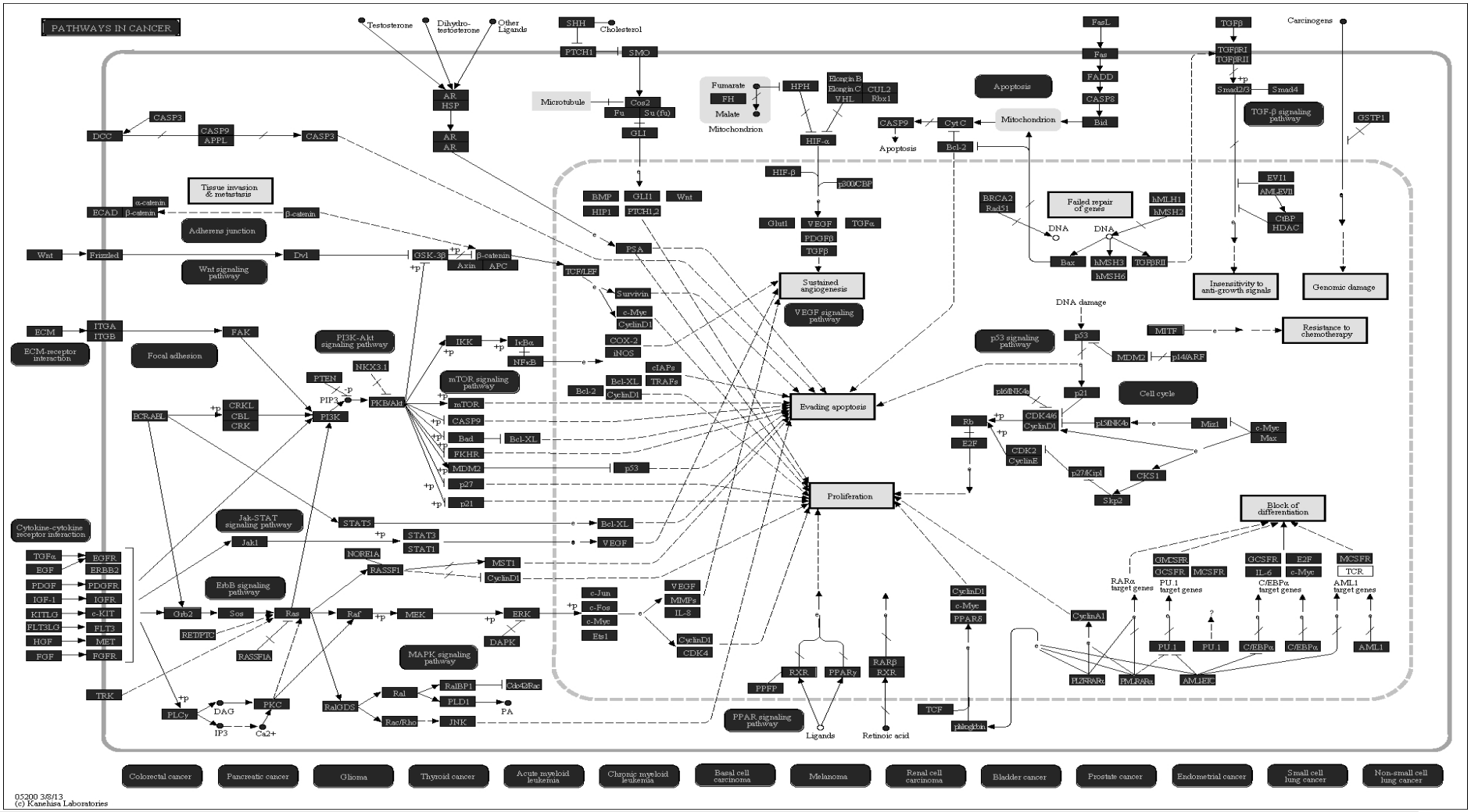
Appendix 26: Pathway Analysis Charts

C. HBV Infection and Tuberculosis Pathway



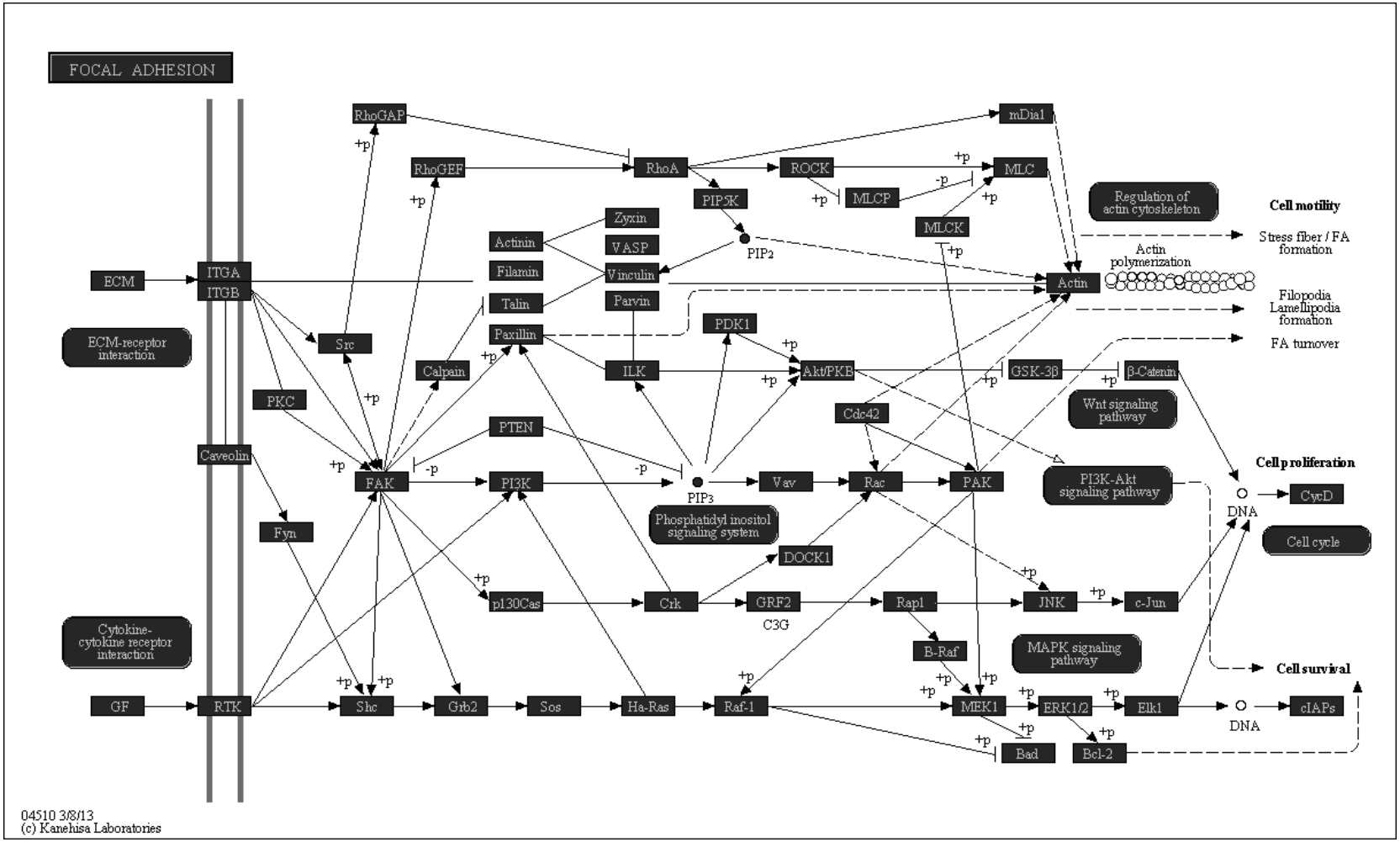
Appendix 26: Pathway Analysis Charts

D. HBV Infection and Cancer Pathway



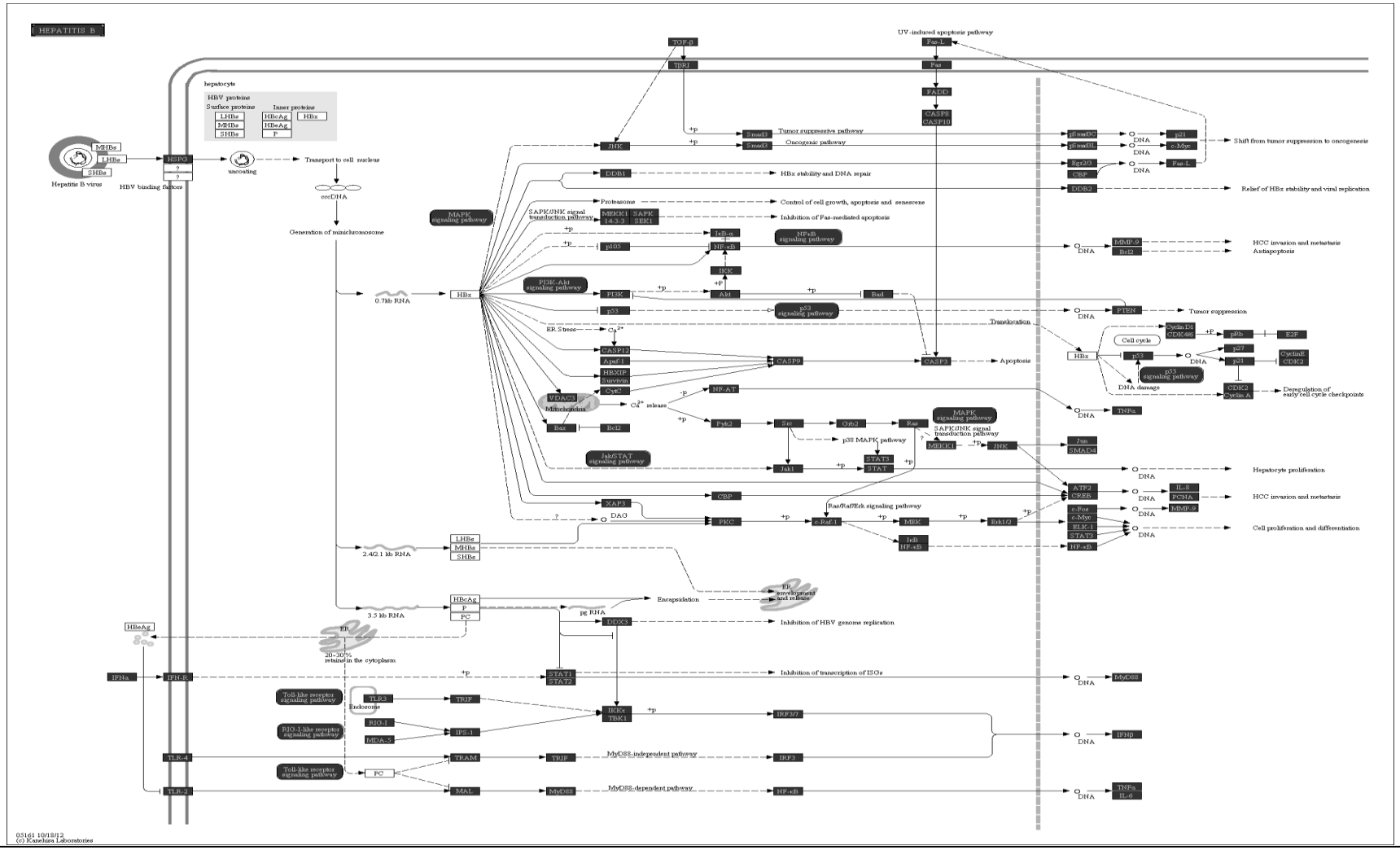
Appendix 26: Pathway Analysis Charts

E. HBV Infection and Cell Adhesion Pathway



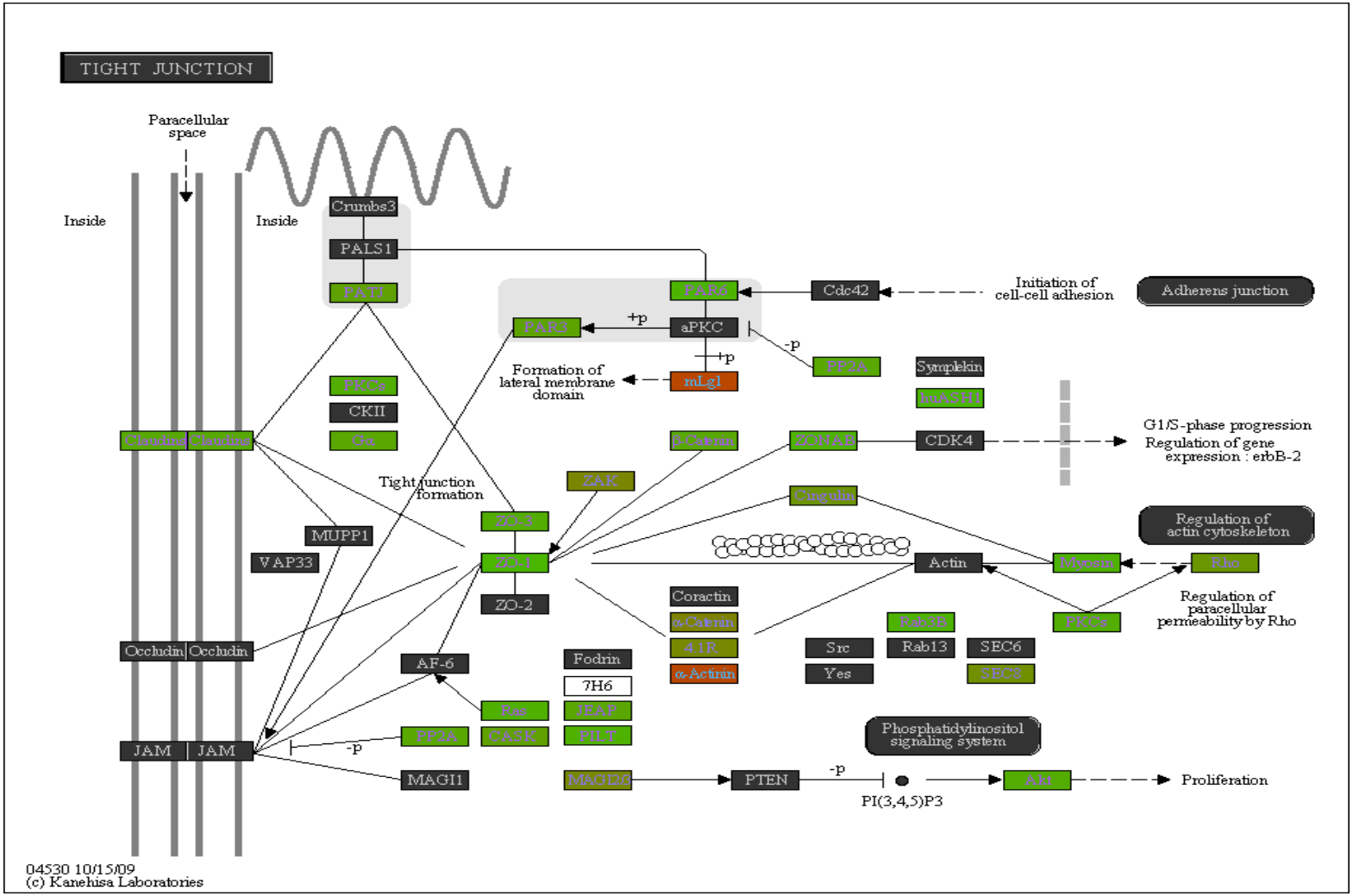
Appendix 26: Pathway Analysis Charts

F. HBV Infection Pathway

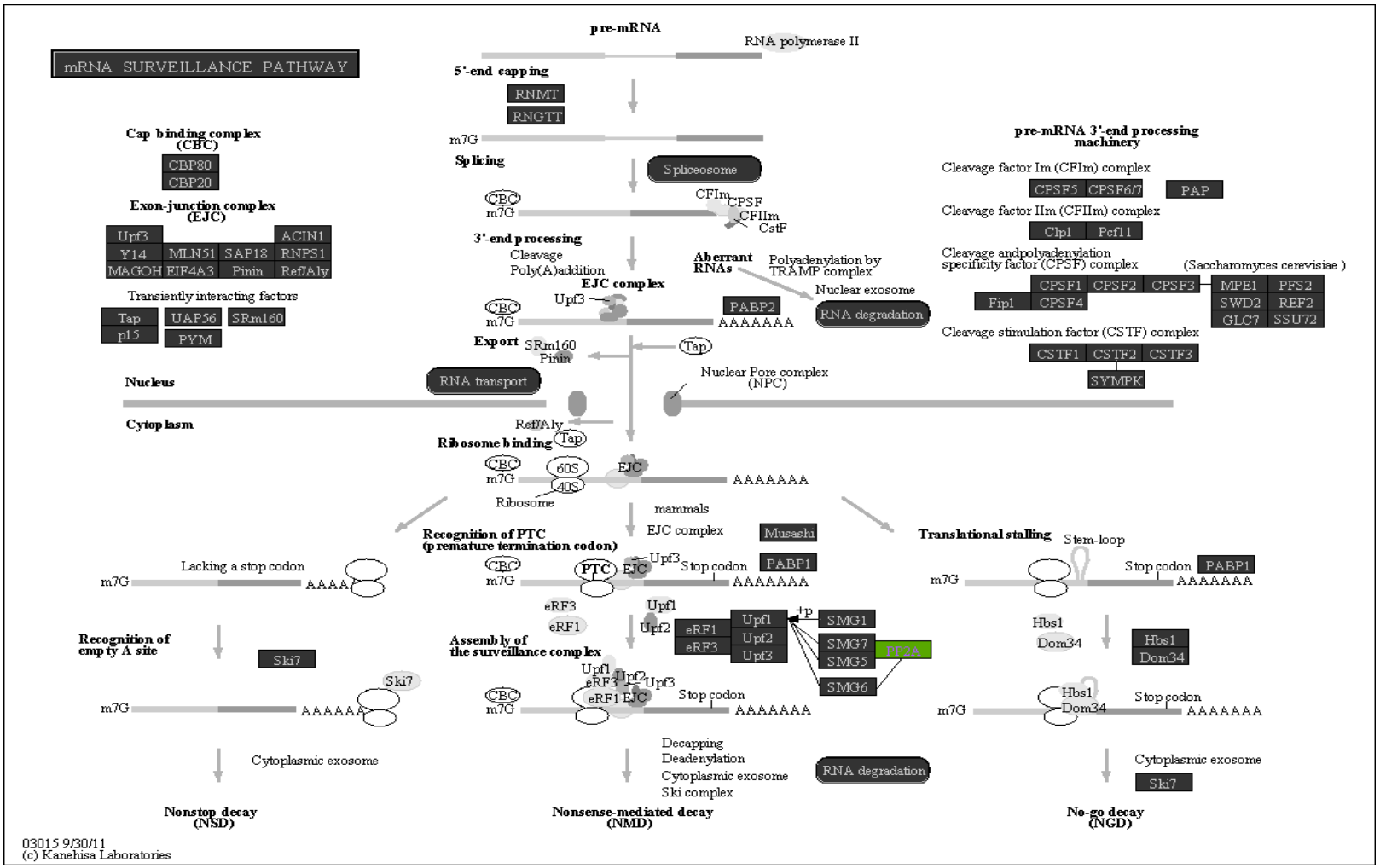


Appendix 26: Pathway Analysis Charts

G. HBV Infection and Tight Junction Pathway

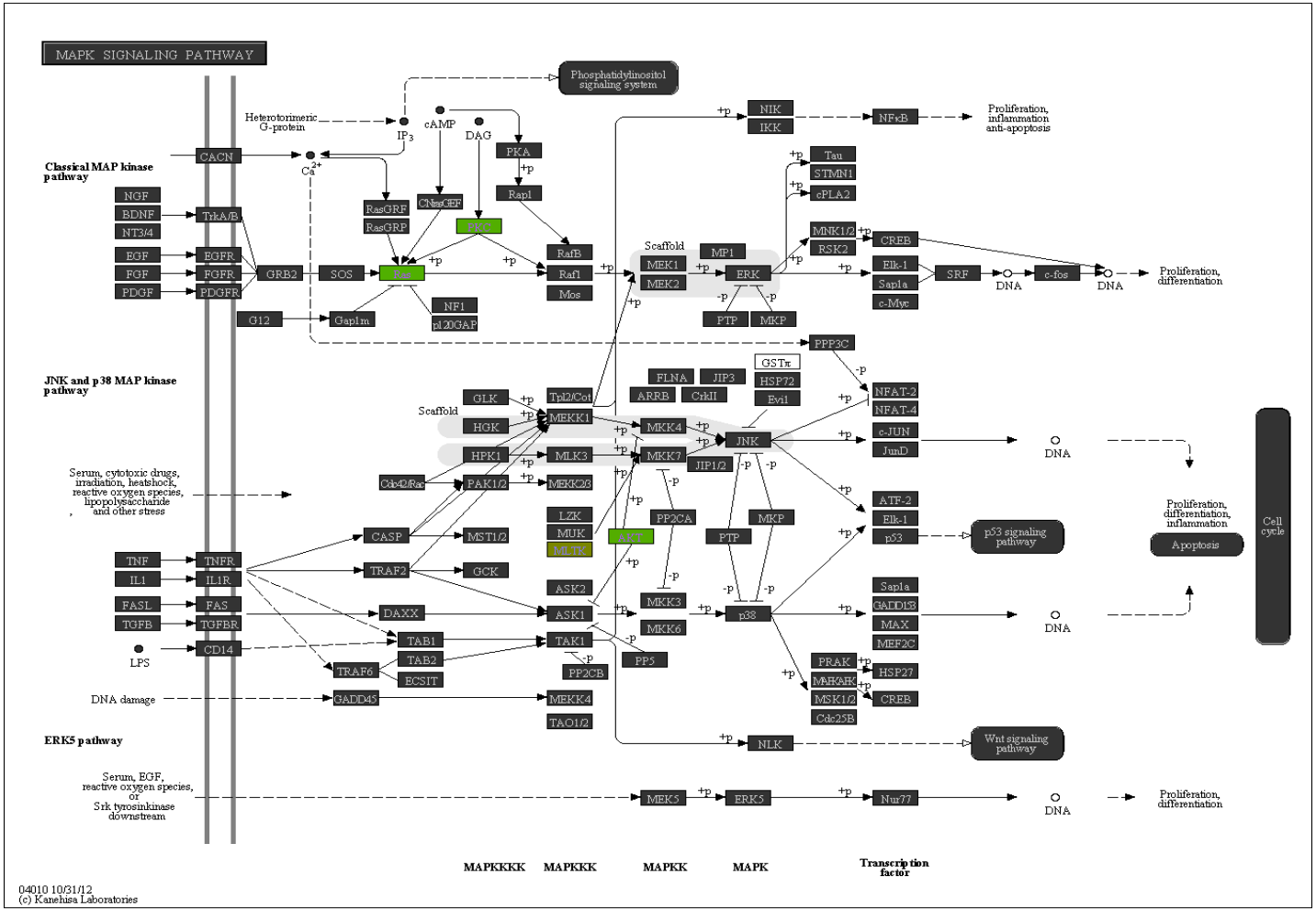


H. HBV Infection and mRNA Surveillance Pathway

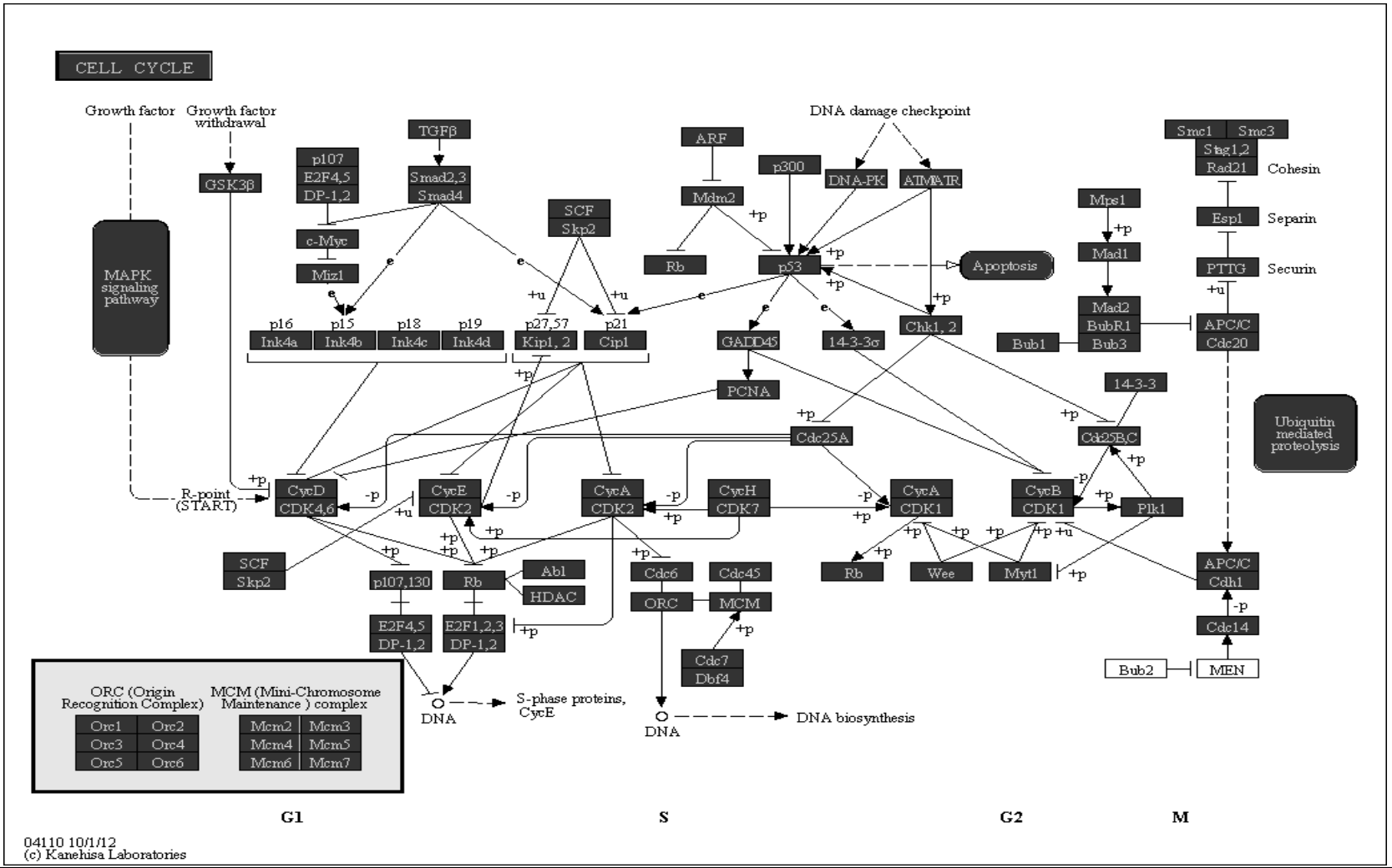


Appendix 26: Pathway Analysis Charts

I. HBV Infection and MAP Kinase Pathway

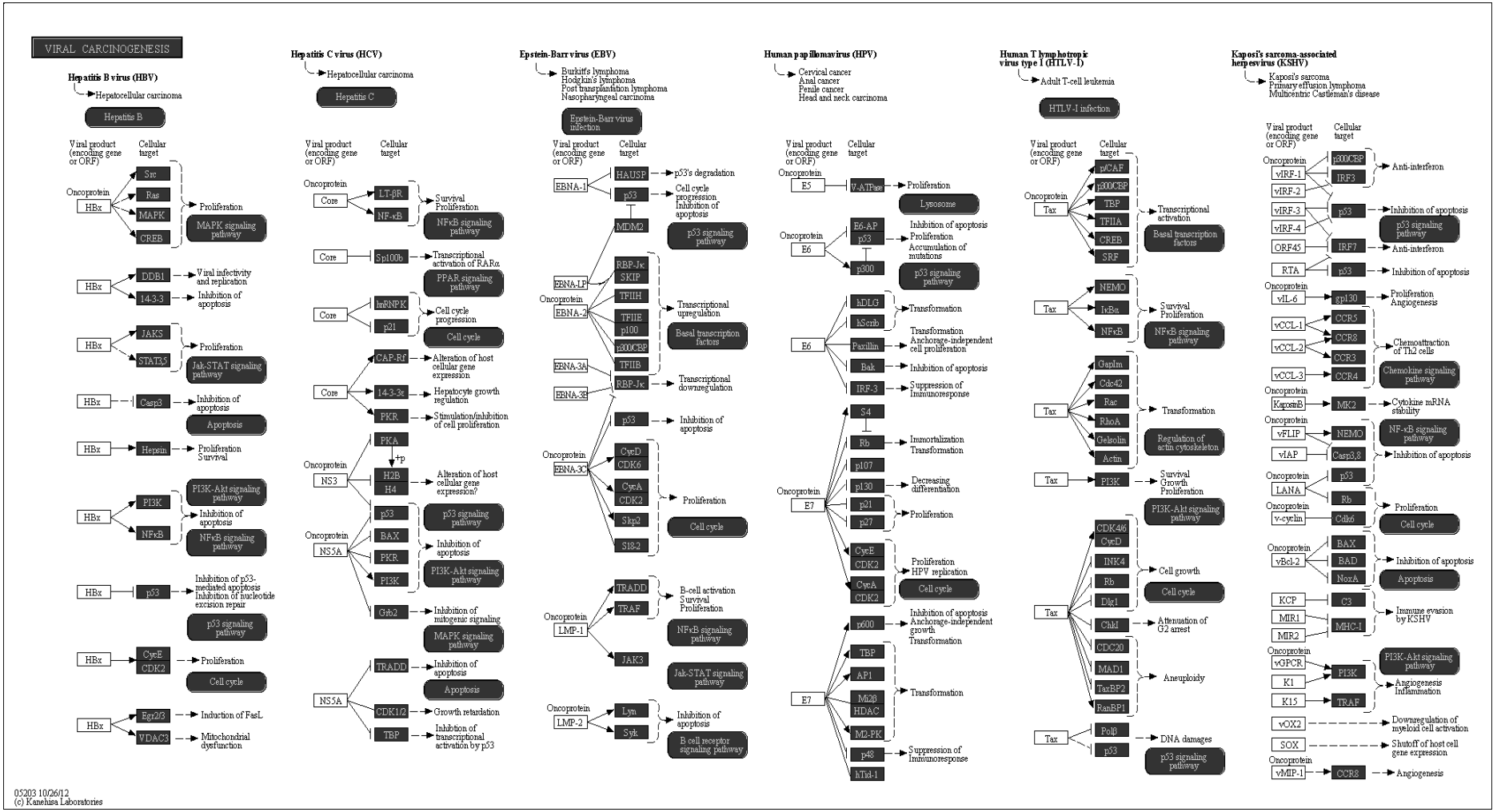


J. HBV Infection and the Cell Cycle Pathway



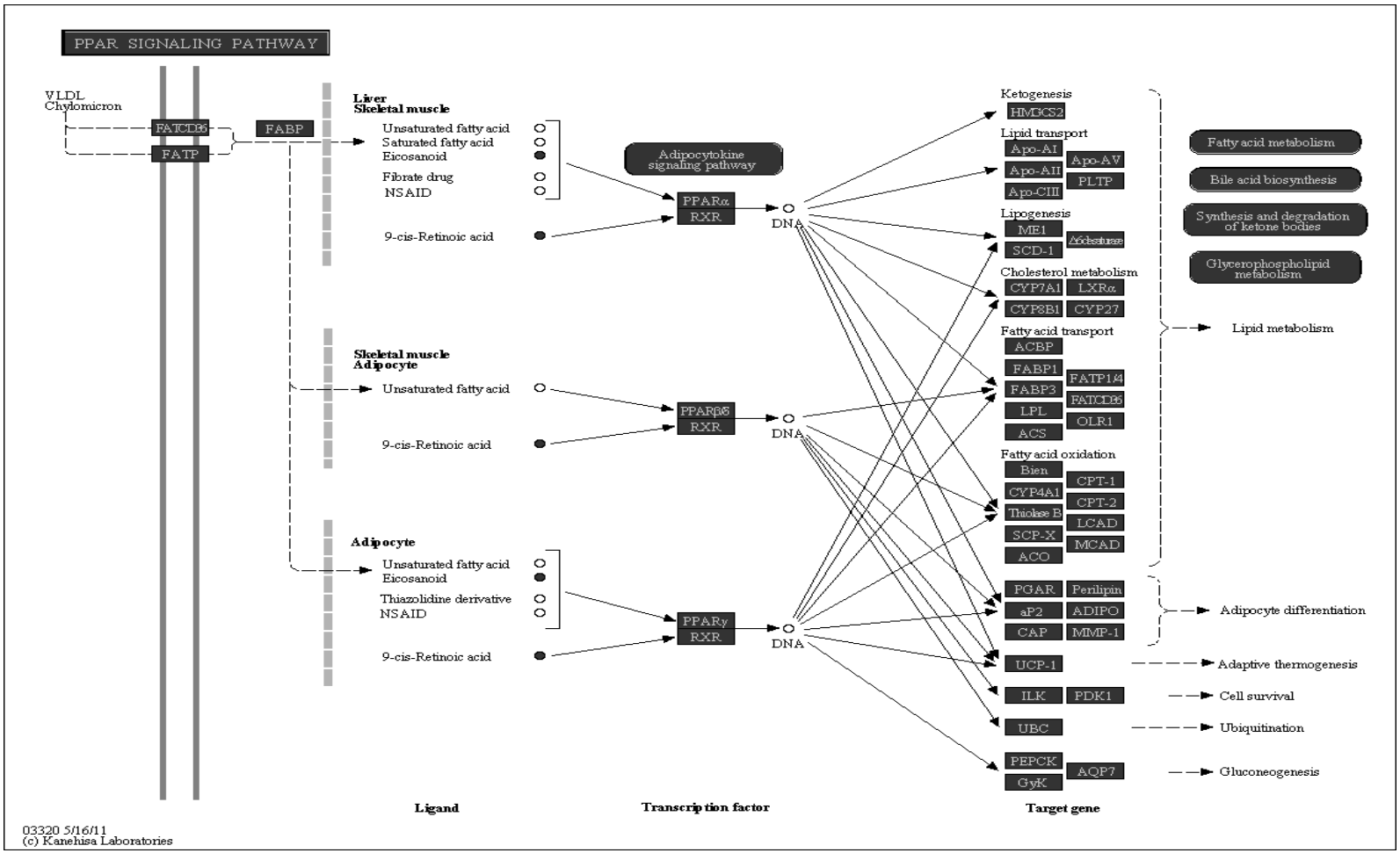
Appendix 26: Pathway Analysis Charts

K. HBV Infection and Viral Carcinogenesis Pathway

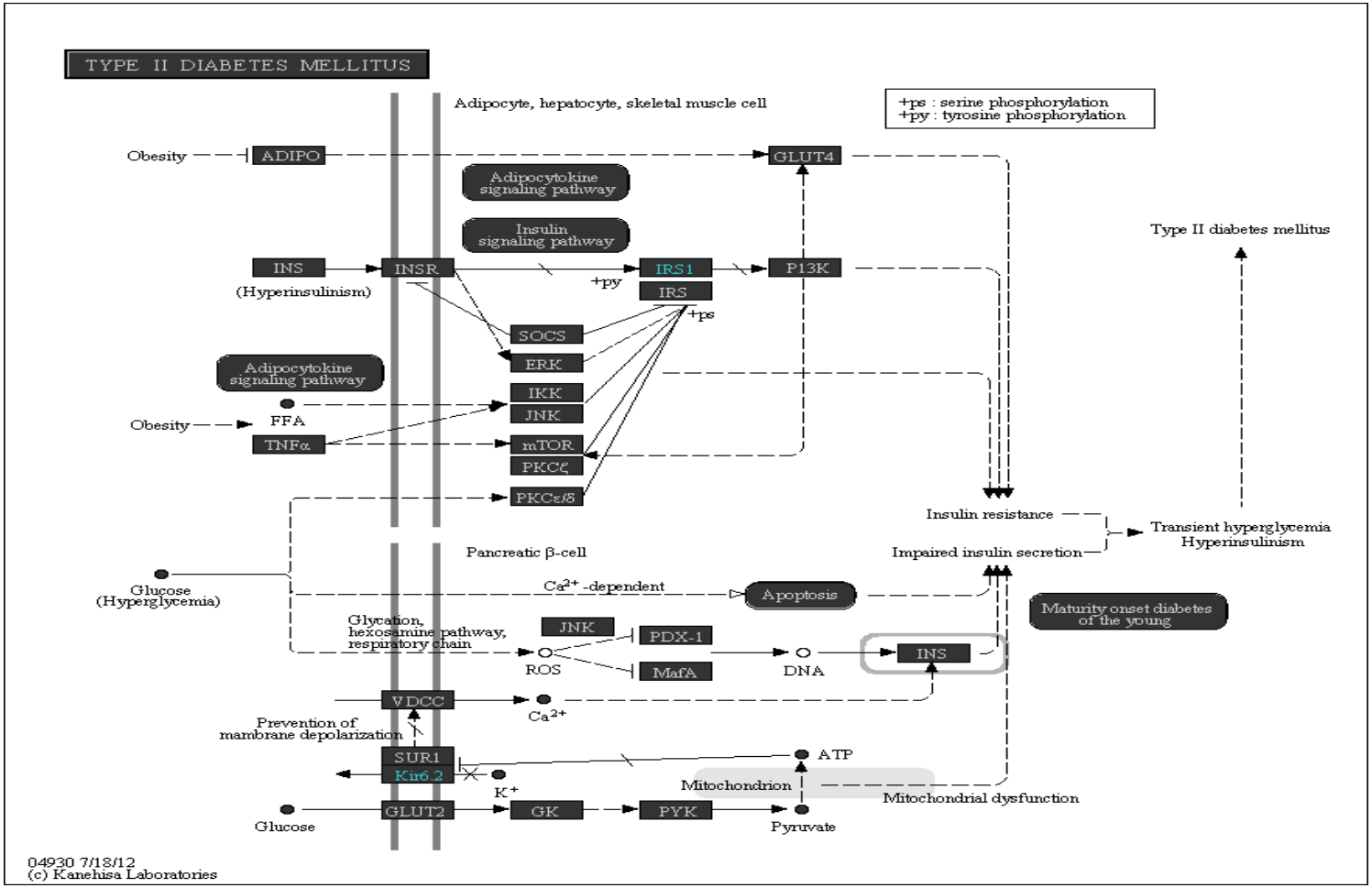


Appendix 26: Pathway Analysis Charts

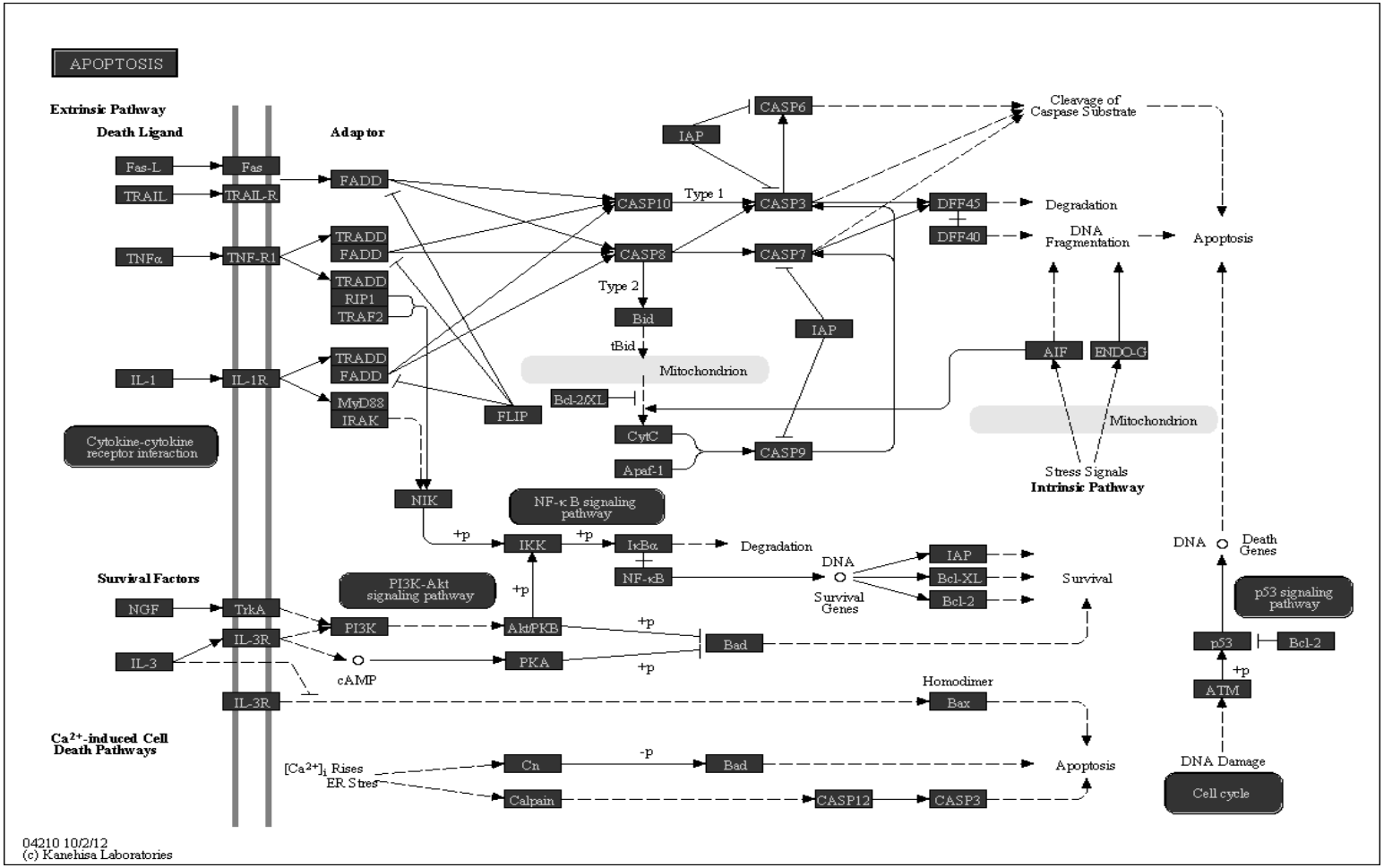
L. HBV Infection and PPAR Gamma Signalling Pathway



M. HBV Infection and Type 2 Diabetes Mellitus Signalling Pathway

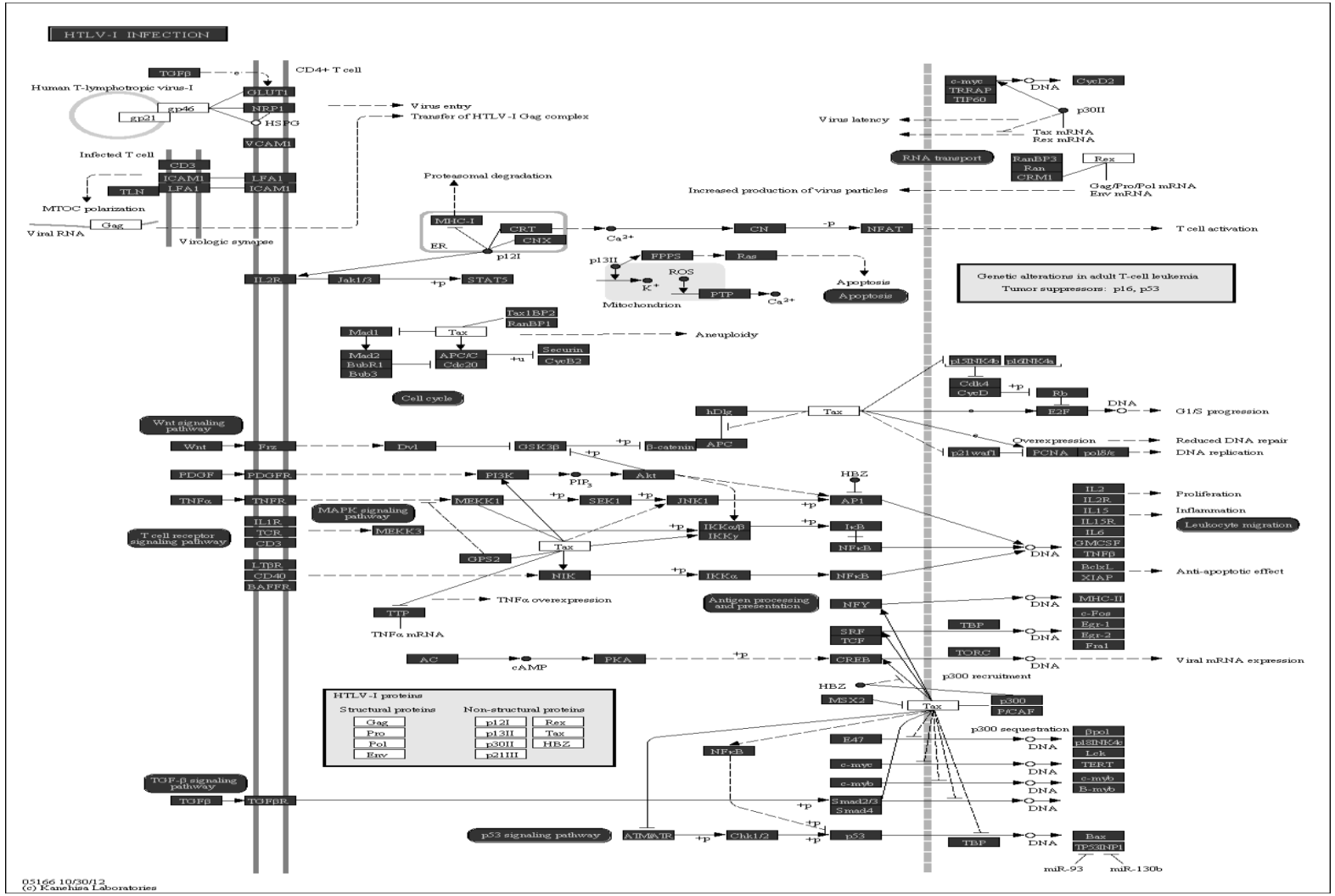


N. HBV Infection And Apoptosis Pathway



Appendix 26: Pathway Analysis Charts

O. HBV Infection and HTLV-1 Infection Pathway



Appendix 26: Pathway Analysis Charts

APPENDIX 27:

ETHICS APPROVAL LETTER

APPENDIX 28:

PATIENT CONSENT FORM

CONSENT FORM: HBV Epigenetic Changes Study

University of Cape Town Health Sciences Faculty Research Ethics Committee Reference Number:

Patient Hospital Number:

Title of project:
Investigation of chronic hepatitis B virus infection induced genome epigenetic changes using DNA immunoprecipitation and microarray techniques to identify promoter DNA methylation changes in patients with chronic HBV infection

Name of Researcher Taking Consent:

I confirm that I have read and understand the information sheet dated February 2010, version3 for the above study and have had the opportunity to ask questions

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected

I understand that sections of any of my medical notes may be looked at by responsible individuals from the Liver Research Centre who are participants in this study or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

I agree to take part in the above study.

- Name of Patient.....

  Signature..... Date.....
- Name of person taking consent .....

  Signature..... Date.....
- Name of Researcher.....

  Signature..... Date.....

NB: Three copies should be signed, for (1) patient, (2) researcher, (3) hospital notes

Contact Details For Further Information:

Dr BN Hairwadzi: Department of Medicine, Liver Clinic, Groote Schuur Hospital, Old Main Building, Observatory Cape Town 7925. **Telephone:** 021 406 6190 / 6394 **Facsimile:** 021 448 6815.

UCT Human Research Ethics Committee: Room E52-24 Groote Schuur Hospital, Old Main Building, Observatory, Cape Town 7925. **Telephone:** 021 406 6338 **Facsimile:** 021 460 6411.

Consent for DNA Storage and Analysis: HBV Epigenetic Changes Study

If any of the **DNA** I have provided for this research project is unused or leftover when the project is completed (Tick **one** choice from each of the following boxes)

- ☐ I wish my **DNA sample** to be destroyed immediately
- ☐ I want my **DNA sample** to be destroyed after 20 years
- ☐ I give permission for my **DNA sample** to be stored indefinitely

AND (if sample is to be stored)

- ☐ I give permission for my **DNA sample** to be stored and used in future research but only on the same subject as the current research project: **HBV epigenetic changes study**
- ☐ I give permission for my **DNA sample** to be stored and used in future research of any type which has been properly approved
- ☐ I give permission for my **DNA sample** to be stored and used in future research except for research about:_____

AND

- ☐ I want my identity to be removed from my **DNA sample**
- ☐ I want my identity to be kept with my **DNA sample**

I have read the information, or it has been read to me. I have had the opportunity to ask questions about it and my questions have been answered to my satisfaction. I consent voluntarily and understand that I have the right to withdraw my consent without affecting the current research study or my medical care.

Print Name of Participant: _____

Signature of Participant: _____

Date: _____
Day/Month/Year

If illiterate
A literate witness must sign (if possible this person should be selected by the participant and should have no connection to the research team). Participants who are illiterate should include their thumb print as well.

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness: _____ **AND** **Thumb print of participant**

Signature of witness: _____

Date: _____
Day/Month/Year

I have accurately read or witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print Name of Researcher: _____

Signature of Researcher: _____

Date: _____
Day/Month/Year

Copy provided to participant :_____**(Initialled by researcher).**

Appendix 28: Patient Consent Form