CHARACTERIZATION OF MUTANTS AND SPLICE VARIANTS OF HEPATITIS B VIRUS ISOLATED FROM SOUTH AFRICAN BLACK HEPATOCELLULAR CARCINOMA PATIENTS.

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Thesis submitted in compliance for the requirements for the degree of Doctor of Philosophy in the Faculty of Health Sciences at the University of the Witwatersrand

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DECLARATION

I, Michelle Skelton declare that this thesis is my own work. It is be being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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……..day of…….(month), 2009

DEDICATION

I dedicate this thesis to my dear and late grandparents

Lydia Deist, Mary Skelton and Frederick Deist.

PRESENTATIONS

1. Conference: Cancer Research UK Beatson International Cancer Conference, pg 115, Scotland, UK.

   Poster: Michelle Skelton, Gerald C Kimbi, Anna Kramvis and Michael C. Kew (2005). Characterization of hepatitis B virus mutants in hepatocellular carcinoma in southern Africa.
2. Workshop: South African-Swedish Research Partnership (SIDA/NRF) Workshop Phylogenetic analysis of Hepatitis viruses found in Southern Africa relative to those from other geographic regions of the world. UWC, Cape Town, South Africa (2006).

   Presentation: Characterization of hepatitis B virus mutants in hepatocellular carcinoma in southern Africa.
3. MRC review of the Molecular Hepatology Research Unit in Johannesburg (27 June 2006).

   Presentation: Characterization of hepatitis B virus mutants in hepatocellular carcinoma in southern Africa.
4. Symposium: Hepatitis B Virus Genotypes ~ from an academic question to the clinic (July 2008).

   Presentation: Subgenotype A1 splice variants and mutants isolated from serum of South Africa hepatocellular carcinoma patients.

ABSTRACT

Hepatitis B virus (HBV) infection is endemic in Africa. As many as 98% of black Africans are infected during their lives and about 10% (65 million) have chronic HBV infection, which is the cause of 70-80% of all hepatocellular carcinoma (HCC) cases. Despite this high prevalence of HBV and the high incidence of HCC in Africa, relatively few complete HBV genomes from African HCC cases have been deposited in international data bases. In order to gain a clearer understanding of the role of genetic variants and mutants in the development of HCC, the complete genomes of HBV isolated from southern African HCC patients were amplified and molecularly characterized. HBV DNA was extracted from the serum forty HBsAg-positive HCC patients. Twenty six complete genomes were successfully amplified, cloned and sequenced from nine HCC patients.

Phylogenetic analyses of the complete genomes and the individual open reading frames of HBV isolates from the HCC patients, led to the classification of all the isolates within subgenotype A1. No isolates belonging to subgenotype A2 and genotype D were identified, even though these genotypes/subgenotypes have been shown to circulate in South Africa. Three patients contained the uncommon combination of serological subtype *ayw*1 in the subgenotype A1 strain. This combination has been found previously in South Africa and the Phillipines.

Seventy-eight percent of the patients carried HBV strains with the double basic core promoter (BCP) mutation (1762T/1764A), previously shown to reduce HBeAg expression. Furthermore, complete genome sequence analysis has revealed a complex combination of mutations, which include at least three or five of these residues 1753C1762T1764A1766T1768A1809T1812T occurring as the dominant

HBV strains isolated from 5/9 HCC patients. These mutations have previously been shown to regulate gene expression at various levels, to enhance viral replication and simultaneously decrease HBeAg expression.

All five HBV genomes isolated from one patient contained novel complex BCP rearrangements, which introduced 2 HNF1 and 1 putative HNF3 transcription factor binding sites. These mutations can enhance viral replication and simultaneously abolish HBeAg expression at a transcriptional level. Furthermore, truncated core proteins would be expressed from 4/5 isolates and none would express wild-type HBx. Several mutations were identified in the pre-S/S genes of 2/5 isolates, which would result in the expression of novel 3' truncated medium surface proteins ($MHBs^t$) and large surface proteins ($LHBs^t$). The majority of the mutations would contribute to hepatocyte pathogenesis and transformation by activating cell proliferating pathways.

Two patients also contained rare HBV variants not previously identified in HBV strains from southern Africa. These included an HBV splice variant and a poly (dA) variant from patient 10 and patient 6, respectively. These variants occurred in combination with other isolates within the respective patients.

The envelope genes were characterised in a total of 18 HCC patients, the pre-S gene of HBV contained deletions in 72% of the patients. Deletions across pre-S1/pre-S2, pre-S2 initiation codon mutations with internal deletions, and S gene nonsense mutations were prevalent. Mutated envelope proteins have been shown to accumulate within the hepatocyte endoplasmic reticulum (ER) and are a characteristic histopathological hallmark of HCC known as ground glass

hepatocytes. HBV induced ER stress has been shown to dysregulate several cell cycle regulatory pathways, which contribute to HCC.

In addition several novel $LHBs^t$ and $MHBs^t$ have been described. These potential transactivators require further investigation. The HBV mutations described in this study have been associated with increased risk for HCC.

Despite the obvious heterogeneity HBV displays within and between patients, there are common characteristics shared between the HBV variants which emerge during the development of HCC. These include the BCP and pre-C (1753C1762T1764A1766T1768A1809T1812T) mutations and the pre-S/S mutations. These mutations are able to affect HBV replication and gene expression, and may work synergistically to promote liver dysfunction and HCC.

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ε	encapsidation signal
aa	amino acid
ALT	Alanine aminotransferase
APC	Antigen presenting cell
ASC	Asymptomatic chronic carrier
AS	Asymptomatic chronic carrier (pertaining to figures)
ASHV	Arctic squirrel hepatitis B virus
bp	base pair
BCP	Basic core promoter
BQW	Best quality water
cccDNA	Covalently closed circular DNA
COR	Cohesive overlap region
COUP-TF	Chicken ovalbumin upstream promoter transcription factor
CURS	Core upstream regulatory sequence
DHBV	Duck hepatitis B virus
DNA	Deoxyribonucleic Acid
DNAML	DNA maximum likelihood
DR1	Direct repeat one
DR2	Direct repeat two
ERK	Extracellular signal-regulated kinase
ENHI	Enhancer one
ENHII	Enhancer two
ER	Endoplasmic reticulum
ESLD	End stage live disease

GGH	Ground glass hepatocyte
GHSV	Ground squirrel hepatitis B virus
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HHBV	Heron hepatitis B virus
HNF	Hepatocyte nuclear factor
IFN	Interferon
IPTG	isopropyl-beta-D-thiogalactopyranoside
kb	kilobase
LB	Luria-Bertini broth
LHBs	Large surface proteins
$LHBs^t$	Truncated large surface proteins
MAPK	Mitogen activated protein kinase
MHBs	Medium surface proteins
$MHBs^t$	Truncated medium surface proteins
mRNA	messenger RNA
Myr	Myristoylation
NEIGHBOR	neighbour-joining
NG	N-glycosylation
NRE	Negative regulatory element
OG	O-glycosylation
ORF	Open reading frame

PCR	Polymerase chain reaction
PK	Protein kinase
PKC	Protein kinase C
Pol	polymerase
Poly A	polyadenylation signal
PPAR α	peroxisome proliferator activated receptor α
PHYLIP	phylogeny inference package
RT	reverse transcriptase
RXR α	retinoid X receptor α
SHBs	Small (major) surface proteins
TBP	TATA binding protein
TP	terminal protein
Top I	Topoisomerase I
TR2	Human testicular receptor 2
URR	Upstream regulatory element
UV	Ultraviolet
WHO	World Health Organization
WHV	Woodchuck hepatitis B virus
WMHBV	Woolly Monkey hepatitis B virus
YMDD	Tyrosine, Methionine, Aspartic acid, Aspartic acid
YIDD	Tyrosine, Isoleucine, Aspartic acid, Aspartic acid
YVDD	Tyrosine, Valine, Aspartic acid, Aspartic acid