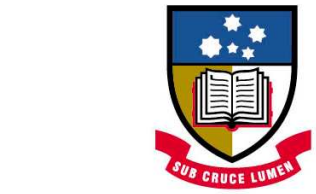

THE UNIVERSITY of ADELAIDE

Clonal proliferation of hepatocytes during chronic hepatitis B virus infection

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B.Sc. (Hons) (Biomedical Science)

A thesis submitted to The University of Adelaide for

The Degree of Doctor of Philosophy

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Abstract

Chronic hepatitis B virus (HBV) infection causes liver disease that can progress to cirrhosis and hepatocellular carcinoma (HCC). Changes in the hepatocyte population that occur from the early immune-tolerant stage of infection to late-stage disease outcomes remain unclear. We hypothesised that some hepatocytes lose HBV antigen expression and escape the HBV-specific immune response, allowing them to undergo clonal proliferation. Clonal proliferation of altered hepatocytes may be a marker of disease progression and may have a direct role in the development of HCC.

Liver tissues from 30 patients were analysed, including patients with early-stage HBV infection, late-stage infection with cirrhosis, or with HCC. Unique virus-cell DNA junctions formed by the integration of HBV DNA into the host cell genome were detected using inverse nested PCR (invPCR). The copy number of unique virus-cell DNA junctions was used as a measure of clonal proliferation of hepatocytes. A computer simulation of a liver undergoing stochastic liver turnover was used to determine if the hepatocyte clones observed by invPCR could have been formed by random chance. Immunohistochemistry for HBV surface antigen (HBsAg) expression and Imaging Mass Spectrometry (IMS) for cellular protein expression were carried out to detect cellular changes that may be associated with clonal proliferation.

Significantly ($p<0.01$) larger clones were observed by invPCR in liver DNA extracts of patients with late-stage HBV-associated disease (≤280000 hepatocytes) compared to patients in early-stage HBV infection (8-1124 hepatocytes). Computer simulations indicated that stochastic turnover could not produce clones of >10000 hepatocytes, suggesting that the hepatocytes that had formed large clones had a survival advantage. No significant difference in the extent of clonal proliferation was observed in foci of HBsAg-positive and -negative hepatocytes isolated by laser-microdissection. Heterogeneous expression of cellular proteins was detected using IMS in hepatocytes with apparently normal histology.

These results indicate that clonal proliferation of hepatocytes with survival advantage does occur in the hepatocyte population during chronic HBV infection and can be detected before histological changes are evident in the hepatocytes of patients with both early- and late-stage disease. Consistent with our hypothesis, larger hepatocyte clones were associated with disease progression. The cause of the clonal proliferation remains unknown. Contrary to our

hypothesis, loss of HBsAg expression was not associated with increased clonal proliferation, suggesting that escape from HBsAg-specific immune attack is not a survival advantage. While not investigated in this thesis, the loss of expression of other HBV antigens may provide a survival advantage. Heterogeneous cellular protein expression suggests that hepatocyte phenotype has been altered in some hepatocytes. However, we could not show using invPCR approaches that the foci of hepatocytes with altered cellular protein expression were clonal.

In conclusion, this research has provided groundwork in determining the relationship between the clonal proliferation of hepatocytes, altered hepatocyte phenotype and HBV-associated disease progression. Further studies into the molecular causes of clonal proliferation of hepatocytes with survival advantages could elucidate pathways of HBV-associated disease progression and novel ways to curb the evolution of the hepatocyte population to a less pathogenic state.

Declaration

NAME: Thomas Tu PROGRAM: Doctor of Philosophy (Ph.D.)

I declare that no material from this work has been submitted for the award of any other degree or diploma in the University of Adelaide or any other tertiary institution. To the best of my knowledge and belief, this work contains no material previously published or written by another person, except where due reference has been made in the text.

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Thomas Tu **October, 2012**

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“A poet once said, "The whole universe is in a glass of wine." We will probably never know in what sense he meant that, for poets do not write to be understood. But it is true that if we look at a glass of wine closely enough we see the entire universe. There are the things of physics: the twisting liquid which evaporates depending on the wind and weather, the reflections in the glass, and our imagination adds the atoms. The glass is a distillation of the Earth's rocks, and in its composition we see the secrets of the universe's age, and the evolution of stars. What strange arrays of chemicals are in the wine? How did they come to be? There are the ferments, the enzymes, the substrates, and the products. There in wine is found the great generalization: all life is fermentation. Nobody can discover the chemistry of wine without discovering, as did Louis Pasteur, the cause of much disease. How vivid is the claret, pressing its existence into the consciousness that watches it! If our small minds, for some convenience, divide this glass of wine, this universe, into parts — physics, biology, geology, astronomy, psychology, and so on — remember that Nature does not know it! So let us put it all back together, not forgetting ultimately what it is for. Let it give us one more final pleasure: drink it and forget it all!”

- Richard P Feynman, Volume I, 3-10, The relation of Physics to other sciences

Publications and presentations resulting from this thesis

Manuscripts in preparation

Tu, T., Mason, W.S., Low, H.C., Clouston, A., Stroeher, U., and Jilbert A.R.
Clonal proliferation of hepatocytes is associated with disease progression in chronic hepatitis B virus infection.

Tu, T., Gustafsson, J., Ho, Y.Y., Mason, W.S., Clouston, A., Stroeher, U., and Jilbert A.R.
Heterogeneous viral and cellular protein expression in hepatocytes during chronic hepatitis B virus infection – analysis by imaging mass-spectrometry.

Tu, T., Ruszkiewicz, A., Clouston, A., and Jilbert A.R.
Liver cell coexpression of hepatitis B surface antigen and cytokeratin 19 during chronic HBV infection.

Oral presentations at international conferences

Tu, T., Mason, W.S., Low, H., Clouston, A., Stroeher, U., and Jilbert A.R. (2012)
Clonal proliferation of hepatocytes in chronic hepatitis B virus infection.
14th International Symposium on Viral Hepatitis and Liver Disease, Shanghai International Convention Centre, 24th June 2012

Tu, T., Mason, W.S., Low, H., Clouston, A., Stroeher, U., and Jilbert A.R. (2010)
Clonal proliferation of hepatocytes in chronic hepatitis B virus infection.
The International Meeting for the Molecular Biology of Hepatitis B Viruses, Academia Sinica, Taipei, Taiwan, 12th October 2010

Poster presentations at international conferences

Tu, T., Mason, W.S., Low, H., Clouston, A., Grosse, A., Stroeher, U., and Jilbert A.R. (2009)
Clonal proliferation of hepatocytes in chronic hepatitis B virus infection.
The International Meeting for the Molecular Biology of Hepatitis B Viruses, Vinci International Convention Centre, Tours, Loire Valley, France, 30th August 2009.

Oral presentations at national conferences

Tu, T., Mason, W.S., Low, H., Clouston, A., Stroeher, U., and Jilbert A.R. (2011)
Clonal proliferation of hepatocytes in chronic hepatitis B virus infection.
Australian Centre for HIV and Hepatitis Virology Workshop 2011, Twin Waters Resort, Sunshine Coast, QLD, 1st June 2011.

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Clonal proliferation of hepatocytes in chronic hepatitis B virus infection.
Australian Centre for HIV and Hepatitis Virology Workshop 2009, Crowne Plaza, Terrigal, NSW, 3rd June 2009.

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Clonal proliferation of hepatocytes in chronic hepatitis B virus infection.
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Tu, T., Low, H., Miao, Y., Mason, W.S., and Jilbert A.R. (2007)
Clonal proliferation of hepatocytes in chronic hepatitis B virus infection.
Australian Centre for Hepatitis Virology Workshop 2007, Burnet Institute, Melbourne, 15th May 2007.

Oral presentations at awards presentations

Tu, T., Low, H., Grosse, A., Miao, Y., Mason, W.S., and Jilbert A.R. (2008)
Clonal proliferation of hepatocytes in chronic hepatitis B virus infection.
The Australian Society for Microbiology (SA Branch) 2008 Student Awards Night, Flinders Medical Centre, Adelaide, 26th November 2008.

Poster presentations at national conferences

Tu, T., Mason, W.S., Low, H., Clouston, A., Grosse, A., Stroeher, U., and Jilbert A.R. (2009)
Clonal proliferation of hepatocytes in chronic hepatitis B virus infection.
Australian Society of Microbiology meeting 2009, Perth Convention Exhibition Centre, Perth, 6th July 2009.

Tu, T., Low, H., Miao, Y., Mason, W.S., and Jilbert A.R. (2007)
Clonal proliferation of hepatocytes in chronic hepatitis B virus infection.
4th Australian Virology Group Meeting 2007, Kingfisher Bay Resort, Frazer Island, 9th December 2007.

Oral presentations in a seminar series

Tu, T., Mason, W.S., Low, H., Clouston, A., Stroeher, U., and Jilbert A.R. (2009)
Clonal proliferation of hepatocytes in chronic hepatitis B virus infection.
Medical Infectious Diseases Research Seminar Series, Institute of Medical and Veterinary Science (Now SA Pathology), Adelaide, 27th August 2010.

Tu, T., Mason, W.S., Low, H., Clouston, A., Grosse, A., Stroeher, U., and Jilbert A.R. (2009)

Clonal proliferation of hepatocytes in chronic hepatitis B virus infection.
Infectious Diseases Laboratories Research Seminar Series, Institute of Medical and Veterinary Science (Now SA Pathology), Adelaide, 24th April 2009.

Tu, T., Low, H., Grosse, A., Miao, Y., Mason, W.S., and Jilbert A.R. (2008)
Clonal proliferation of hepatocytes in chronic hepatitis B virus infection.
Infectious Diseases Laboratories Research Seminar Series, Institute of Medical and Veterinary Science (Now SA Pathology), Adelaide, 13th June 2008.

Bold represents presenting author

Awards resulting from this thesis

Invited Guest Speaker Honorarium from 14th International Symposium on Viral Hepatitis and Liver Disease, valued at USD $1500 (Awarded in 2012)

Integrated Science Young Achievement award at Australian Centre for HIV and Hepatitis Virology Workshop 2011, valued at AUD $500 (Awarded in 2011)

Student Travel Grant Award, International Meeting for Molecular Biology of HBV, valued at USD $800 and USD $1100 (Awarded in 2009 & 2010)

ASM SA Branch Student Award, valued at AUD $1500 (Awarded in 2008)

Abbreviations

Abbreviation	Full Meaning
%GC	Guanosine and Cytosine content (expressed as a percentage)
~	Approximately
<	Less than
=	Equal to
>	Greater than
≥	Greater than or equal to
°C	degree(s) Celsius
µg	Microgram(s)
µL	Microlitre(s)
µM	Micromolar
µm	Micrometre(s)
5'-CG-3'	Cytosine-guanadine dinucleotide sequences
Ab	Antibodies
ABC	Avidin-Biotin Complex
ABI	Applied Biosystems
ACN	Acetonitrile
AFP	Alpha Fetoprotein
ALT	Amino liver transferase
APC	Adenomatous Polyposis Coli
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BRDU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
cccDNA	Covalently closed circular DNA
CCl_4	Carbon tetrachloride
C_{die}	Cell nominated for cell death
CHCA	α-cyano-4-hydroxycinnamic acid
CK19	Cytokeratin 19
C_{live}	Cell nominated to replace dying cell
cm	Centimetre(s)
CNTF	Ciliary Neurotrophic Factor
COBRA	Combined Bisuphite and Restriction Analysis

COX	Cytochrome c oxidase
CPM	Counts per minute
CV	Central Vein
Da	Dalton(s)
DAB	3,3'-Diaminobenzidine tetrahydrochloride
dCTP	Deoxycytidine
DHBV	Duck hepatitis B virus
DMNT	DNA methyltransferase
DNA	Deoxyribonucleic acid
DOP	Degenerate Oligonucleotide Primer
DR	Direct repeat sequence
dslDNA	Double-stranded linear DNA
DTT	Dithiothreiatol
E. coli	*Escherichia coli*
EAA	Ethanol acetic acid
EDTA	Ethylene-diamine-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic Reticulum
EST	Database of Expressed Sequence Tags
EtOH	Ethanol
F	Frequency
FA	Formic Acid
FAH	Foci of altered hepatocytes
FCCC	Fox Chase Cancer Center
FH	Fulminant Hepatitis
g	Gram(s)
GGH	Ground Glass Hepatocytes
H&E	Haematoxylin & eosin
H2O	Water
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HBx	Hepatitis B x protein
HCC	Hepatocellular carcinoma

HCl	Hydrochloric acid
HCV	Hepatitis C Virus
HPLC	High-pressure liquid chromatography
HPLC-nESI-LTQ	High Pressure Liquid Chromotography Electrospray Ionisation
Orbitrap MS	Linear Ion Trap Orbitrap Mass Spectrometry
hr	Hour(s)
HREC	Human Research Ethics Committee
hTERT	Human Telomere Reverse Transcriptase
ID	Unique identifier
IMS	Imaging Mass-Spectrometry
IMVS	Institute of Medical and Veterinary Science
invPCR	Inverse nested PCR
kbp	Kilobase pair
kDa	Kilodalton(s)
L	Litre(s)
*lac*Z	Beta-galactosidease gene
LB	Luria broth
LCC	Large cell change
LHBs	Large Hepatitis B surface antigen
LINE	Long interspersed elements
LTQ	Linear Ion Trap
M	Molar
m/z	Mass:Charge ratio
mA	Milliampere(s)
MALDI	Matrix-assisted laser-desorption/ionisation
mg	Milligram(s)
MgCl2	Magnesium chloride
MHBs	Medium Hepatitis B surface antigen
MHC	Major histocompatability complex
min	Minute(s)
mL	Millilitre(s)
mM	Millimolar
mm	Millimetre(s)
MPN	Most Probable Number
mRNA	Messenger Ribonucleic Acid

MS	Mass spectometry
MW	Molecular weight
NaCl	Sodium chloride
NB	Needle Biopsy
NBT	Nitro blue tetrazolium chloride
NCBI	National Center for Biotechnology Information
ND	Not Detected
NEB	New England Biolabs
nESI	Nano-electrospray ionisation
NHEJ	Non-homologous end joining
NHL	Normal human liver
nm	nanometre(s)
NP40	Nonidet-P40
nt	Nucleotide(s)
O/N	Overnight
ORF	Open reading frame
PAS-D	Periodic acid schiff - diastase
PBS	Phosphate-buffered saline
PCA1	Procollagenase alpha 1
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PD-1	Programmed Death 1
PD-1L	Programmed Death-1 Ligand
PEN	Polyethylene Napthalate
pgRNA	Pregenomic RNA
PK	Proteinase K
pM	Picomolar
pol	Hepatitis B polymerase protein
PT	Portal tract
qPCR	Quantitative Polymerase Chain Reaction
R	Resistant
RB1	Retinoblastoma-1
rcDNA	Relaxed circular DNA
RE	Restriction enzyme(s)
RI	Replicative intermediates

RNA	Ribonucleic acid
rnd	Random number
RP-HPLC	Reverse Phase High Pressure Liquid Chromatography
rpm	Revolutions per minute
RT	Room temperature
s	Second(s)
S	Sensitive
S/MAR	Structural and Matrix Attachment Regions
S/N	Supernatant
SA	Survival advantage
SA_{die}	Survival advantage of the cell nominated for cell death
SA_{live}	Survival advantage of the cell nominated to replace dying cell
SCC	Small cell change
SD	Standard Deviation
SDS	Sodium dodecyl sulphate
SHBs	Small Hepatitis B surface antigen
SINE	Short interspersed elements
SSC	Saline sodium citrate
TAE	Tris-acetate EDTA
TE	Tris-EDTA
TGF	Tumour Growth Factor
Tm	Melting temperature
TO	Turnover(s)
TOF	Time of Flight
TOF/TOF	Tandem Time of Flight
TRF1/2	Telomere Repeat Binding Factor1/2
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
U	unit(s)
ULN	Upper limit normal
UV	Ultraviolet
V	Volt(s)
WHO	World health organisation
WHV	Woodchuck hepatitis virus
X-Gal	Bromo-chloro-indolyl-galactopyranoside
α-HBcAb	Antibodies specific against hepatitis B core antigen

α-HBeAb	Antibodies specific against hepatitis B e antigen
α-HBsAb	Antibodies specific against hepatitis B surface antigen
ε	Epsilon region